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MULTIPLE SITE PHOSPHORYLATION OF THE CARBOXYL TERMINAL REGION OF DYSTROPHIN

BY

CARRIE SIMONE SHEMANKO ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA
FALL 1993

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August 30, 1993

Don't hang on to things too hard,

because nothing ever stays the same.

Grandpa
(Glen Evans)

UNIVERSITY OF ALBERTA

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July 29 , 1993

ABSTRACT

Dystrophin is the 427-kDa protein product of the Duchenne muscular dystrophy gene. The function of this protein remains to be elucidated. Deletions of the carboxyl terminal region of dystrophin result in a severe form of Duchenne muscular dystrophy and suggest that this domain of the protein plays an important role in the pathology of the disease. In order to study this carboxyl region of the molecule, we have constructed dystrophin fusion proteins using glutathione-S-transferase and the carboxyl 201 amino acids of dystrophin (amino acids 3485-3685). Based upon the possibility that dystrophin may behave as a cytoskeletal protein, I investigated whether the dystrophin fusion protein is a substrate for phosphorylation, as are many cytoskeletal proteins. In this study, I have identified three different protein kinase activities in soluble skeletal muscle extracts which phosphorylate the carboxyl terminus of dystrophin. 1) Kinase XA is Ca^{++} activated, positively regulated by protein phosphatases 1 and/or 2A, and phosphorylates the dystrophin carboxyl terminus on a serine residue(s) between amino acids 3485-3667. 2) Kinase XB is active in the absence of Ca^{++}, and phosphorylates the dystrophin carboxyl terminus within at least the last 18 amino acids (3668-3685). 3) p44erk1 mitogen-activated protein kinase phosphorylates the carboxyl terminus on a threonine residue(s) between amino acids 3485-3667. Phosphorylation may regulate the function of this cytoskeletal protein.

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I would like to thank my supervisor, Dr. M. Michalak, for his support and guidance throughout the years.

I would also like to extend my thanks to Jody Busaan, for production and purification of the dystrophin fusion protein GST-CT-1 and all of the anti-dystrophin antibodies, as well as the production and maintenance of the cell cultures. Thanks also goes to Dr. L. Smillie and M. Carpenter for assistance with the phosphoamino acid analysis. Drs. S. Pelech and Dr. J. Sanghera contributed to the MAP kinase section of the project, and their help is greatly appreciated.

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LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

ATP - Adenosine triphosphate
BMD - Beckers muscular dystrophy
cAMP PK - Cyclic adenosine monophosphate dependent protein kinase
cDNA - Complementary deoxyribonucleic acid
CKII - Casein kinase two
CNBr - Cyanogen bromide
DAP - Dystrophin-associated proteins
DEAE - Diethylaminoethyl
DMD - Duchenne muscular dystrophy
DMEM - Dulbecco's modified Eagle's medium
DNA - Deoxyribonucleic acid
DTT - Dithiothreitol
DRP - Dystrophin-related protein
EDTA - Ethylene diaminetetraacetic acid
EGTA - Ethylene glycol-bis(β-aminoethyl ether) N, N, N', N',-tetraacetic acid
ERK - Extracellular regulated kinase
FPLC - Fast protein liquid chromatography
GST - Glutathione-S-transferase
GST-CT-1 - Glutathione-S-transferase-carboxyl terminal-1
γ[^{32}P]ATP - gamma[^{32}phosphate]adenosine triphosphate
HIST - Histone
IPTG - Isopropyl b-D-thiogalactopyranoside
kDa - Kilodalton
KLH - Keyhole limpet haemocyanin
MAP - Mitogen-activated protein kinase
MES - (2-[N-Morpholino]ethanesulfonic acid)
MLR - Microcystin LR
MOPS - 3-(N-morpholino) propane-sulfonic acid
MPK - Meiosis-activated protein kinase
OA - Okadaic acid
PA - Protein A
PBS- Phosphate buffered saline
PKA - Protein kinase A

PKI - Protein kinase inhibitor
PMSF - Phenylmethyl sulfonyl fluoride
PP - Protein phosphatase
SCARMD - Severe childhood autosomal recessive muscular dystrophy
SDS - Sodium dodecyl sulfate
SDS-PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SL - Sarcolemma
TX-100 - Triton-X-100

CHAPTER 1 - INTRODUCTION

I. Duchenne and Becker's Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a common and lethal genetic disorder with an incidence of 1 in 3300 live male births (Emery, 1991). Its clinical manifestations were first documented by Duchenne de Boulogne in the 1860's and Gower in the 1880's. DMD is characterized by progressive muscle degeneration which is apparent at age 4, and leaves the patient no longer ambulatory by 12 years of age. In 1/3 of the cases there is mental retardation as well (Emery, 1988). The onset of death in the late teens or early twenties is usually caused by pulmonary infection and respiratory failure (Vignos, 1977). Becker's muscular dystrophy (BMD) is a related though less severe form of the disease (Koenig *et al.*, 1989), also characterized by progressive muscle weakness and degeneration. The muscle degeneration in both DMD and BMD involves the loss of individual muscle fibers. Before the gene was identified, high serum levels of creatine kinase (Ebashi *et al.*, 1959) were used as an indicator for diagnosis. Other cellular-level characteristics of the disease include high intracellular levels of Ca^{++} (Turner *et al.*, 1988; Franco and Lansman 1990; Fong *et al.*, 1990).

DMD and BMD both result from recessive X-linked defects in the same gene, which is localized to the short arm of the chromosome X (Xp21) (for review and citations within; Monaco and Kunkel, 1987: Worton and Thompson, 1988). The gene encompasses 2.3 million base pairs of DNA and contains at least 70 exons, producing a 14 kb mRNA (Koenig, *et al.*, 1987). It encodes a 427 kDa protein, called dystrophin (Hoffman, *et al.*, 1987), which is either absent in DMD patients, or truncated in patients with BMD (Bushby *et al.*, 1993). The pathology of the muscle necrosis caused by the lack of intact dystrophin is not well understood.

II. Localization of the Dystrophin Protein

Dystrophin is localized to the muscle sarcolemma (SL) membrane, and more specifically to the subsarcolemmal cytoskeleton (Ohlendieck and Campbell, 1991a) in normal, but not in DMD or muscle of *mdx* mice (Zubrzycka-Gaarn *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988; Michalak and Zubrzycka-Gaarn, 1990). The *mdx* mouse is an animal model for DMD. Dystrophin's interaction with the plasma membrane appears to be mediated through a complex of SL glycoproteins (see dystrophin-associated proteins, DAPs, section V-3 this chapter and figure 1-1). Further research has placed dystrophin at three specific domains of the SL membrane, in elements overlying the I bands and M lines with β-spectrin, and along the latitudinal axis of the myofiber like vinculin (Porter *et al.*, 1992); the myotendinous junction (Shimizu *et al.*, 1989, Samitt and Bonilla, 1990, Byers *et al.*, 1991, Peng and Chen, 1992); and areas of the plasma membrane where there are extensive cytoskeleton-membrane interactions such as the acetylcholine receptor-rich postsynaptic membrane of *Torpedo* electric organ (Chang *et al.*, 1989; Jasmin *et al.*, 1990; Yeadon *et al.*, 1991), and the neuromuscular junction in skeletal muscle (Shimizu *et al.*, 1989; Sealock *et al.*, 1991; Byers *et al.*, 1991; Matsumura *et al.*, 1992).

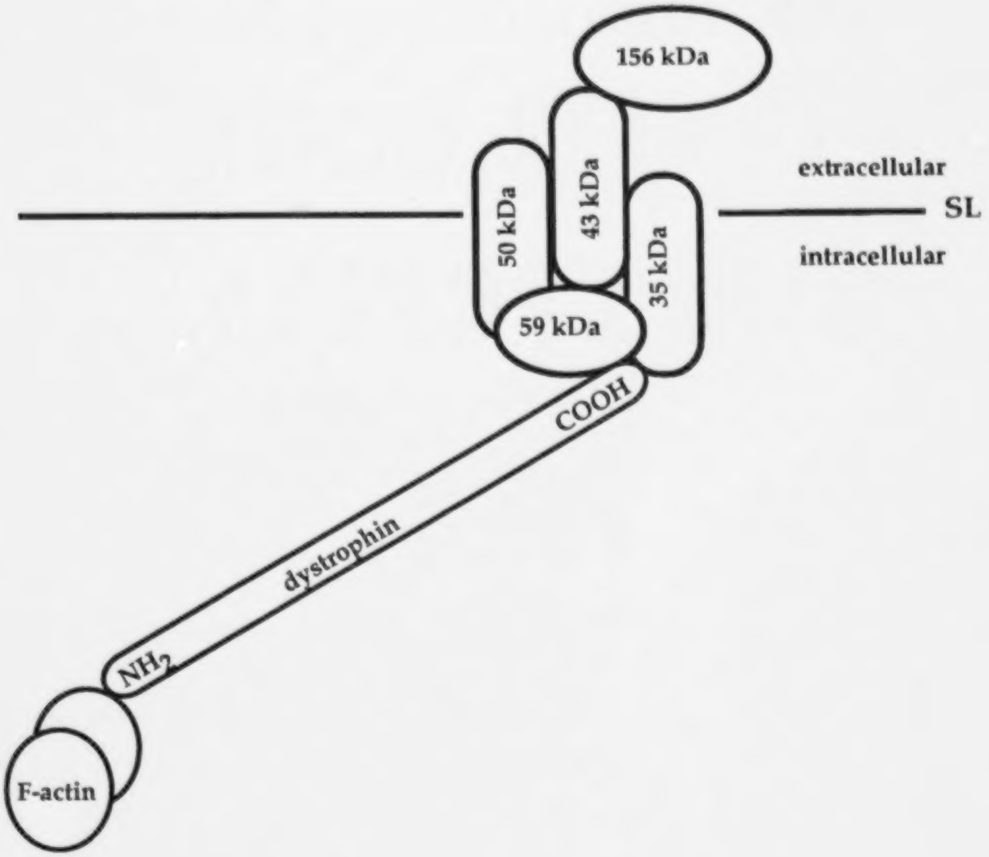
Dystrophin is not restricted to muscle tissue, and has been found in different areas and cells of the brain including the cerebellum (Lidov *et al.*, 1990), glial and neuronal cells (Hoffman, *et al.*, 1987), Purkinje cells (Huard and Tremblay, 1992) and outside the brain in peripheral nerves (Byers *et al.*, 1993).

III. Dystrophin

Dystrophin's function remains to be elucidated. Its amino acid sequence and membrane location suggest that it is a cytoskeletal protein (Koenig *et al.*, 1988). Dystrophin also shares structural and functional similarities with several different proteins. The amino terminus, the first domain, has similarity to α-actinin and binds actin (Hemmings *et al.*, 1992; Way *et al.*, 1992). The second domain, central and rod-shaped, shows similarity to spectrin with its 24 triple-helical repeat structures and four potential hinge regions (Koenig and Kunkel, 1990). The third domain contains a cysteine-rich region with similarity to slime mold α-actinin and contains potential Ca^{++}-binding sites (Koenig *et al.*, 1988). The fourth and last domain at the carboxyl terminus shows protein similarity to the autosomal dystrophin-related protein (DRP) produced from chromosome 6 (Love *et al.*, 1989; Khurana *et al.*, 1990); an 87 kDa protein found in the post-synaptic membrane of the *Torpedo* electric organ and mammalian neuromuscular

Figure 1-1. Dystrophin at the SL in association with membrane glycoproteins

Dystrophin is found at the SL, associated with an extracellular, peripheral protein of 156 kDa, three integral membrane glycoproteins of 50, 43 and 35 kDa, and an intracellular, peripheral 59 kDa protein. A partially characterized 25 kDa integral membrane dystrophin-associated protein is also represented here.


156 kDa
50 kDa
43 kDa
35 kDa
59 kDa
extracellular
SL
intracellular
COOH
dystrophin
NH2
F-actin

junctions (Wagner *et al.*, 1993); a 70-75 kDa protein present in the brain and other nonmuscle tissues, which is a product of an alternative promoter in the DMD gene (Bar *et al.*, 1990; Lederfein *et al.*, 1992; Hugnot *et al.*, 1992), and a 116 kDa alternative protein product present in peripheral nerves.

IV. Dystrophin Related Protein

Dystrophin related protein (DRP) is an autosomal homologue (Love *et al.*, 1989; Khurana *et al.*, 1990; Nguyen *et al.*, 1992;) and has many features the same as dystrophin. Though produced from a gene present on chromosome 6, the protein has a similar molecular weight (395 kDa) and overall structure as dystrophin. Its tissue distribution reveals expression in a wide range of muscle and nonmuscle cells (Love *et al.*, 1991), even in those which do not express dystrophin (Nguyen *et al.*, 1992). Its subcellular localization also reveals similarities to dystrophin, though it is restricted to the neuromuscular junction in normal persons (Nguyen *et al.*, 1991, Pons *et al.*, 1991), and is only found extrajunctionally in the SL in individuals with DMD (Nguyen *et al.*, 1991; Tanaka, *et al.*, 1991). The actin- and DAP- binding regions of dystrophin and DRP are highly conserved between the two proteins (Tinsley *et al.*, 1992). DRP and the dystrophin-associated proteins also immunologically colocalize, and copurify in a manner that suggests that DRP may be able to substitute for dystrophin function in some instances. Its ability to bind the glycoprotein complex, plus its presence in the SL outside the neuromuscular junction in DMD cells, suggests that DRP may compensate for dystrophin deficiency and retain the glycoproteins in extrajunctional regions of the SL (Matsumura *et al.*, 1992b). The function of DRP is unknown.

V. Dystrophin's Protein-Protein Interactions:

V-1. Dystrophin Interacts with Actin

Dystrophin shares sequence similarity with α-actinin, a rod-shaped protein that interacts with filamentous actin. In fact, human dystrophin shares 45% sequence identity and 67% sequence similarity with human α-actinin (Hemmings *et al.*, 1992). The first 233 and/or 246 amino-terminal amino acid residues of dystrophin bind filamentous actin *in vitro* (Hemmings *et al.*, 1992) and *in vivo* (Way *et al.*, 1992). Therefore, dystrophin is both structurally and functionally homologous to α-actinin.

V-2. Dystrophin Contains a Leucine Repeat Motif

Though it is unknown how dystrophin may interact with its various protein partners, dystrophin contains a heptad repeat of leucine residues in the carboxyl terminus. These leucines could be used in protein-protein interactions where the leucines of one protein would interact with the leucines on the opposing protein of a partner protein. The possibility of forming a leucine zipper (Landschultz *et al.*, 1988; Buckland and Wild, 1989) is also shared by those proteins which share carboxyl domain homology with dystrophin, namely DRP and an 87 kDa protein cloned from the *Torpedo* electric organ (Wagner *et al.*, 1993).

V-3. Dystrophin-Associated Proteins, DAPs

Dystrophin is associated with proteins of the SL membrane (Campbell and Kahl, 1989) (figure 1-1). It has been postulated that the dystrophin-associated proteins (DAPs) interact with the cysteine-rich domain and the first half of the carboxyl terminus of dystrophin (Suzuki *et al.*, 1992). This tight, dystrophin-associated complex contains two peripheral proteins of a 156 kDa glycoprotein and 59 kDa protein, and three integral membrane glycoproteins of 50, 43 and 35 kDa . The 59 kDa protein is intracellular, and the 156 kDa glycoprotein is believed to be extracellular (Ervasti and Campbell, 1991). The 156 kDa protein has been found to bind laminin with high affinity (Ibraghimov-Beskrovnaya *et al.*, 1992). Other proteins which may interact with dystrophin include members of the integrin superfamily (Lakonishok *et al.*, 1992); and/or low mass receptors isolated by laminin affinity chromatography (Dickson *et al.*, 1992). Interactions between the SL cytoskeleton and the muscle extracellular matrix are believed to be mediated through dystrophin and the dystrophin-associated glycoproteins (Ervasti and Campbell, 1991).

As members of the dystrophin-associated complex are reduced in cells of DMD or *mdx* muscle (Ervasti *et al.*, 1990; Ohlendieck and Campbell, 1991b; Ibraghimov-Beskrovnaya *et al.*, 1992), it was proposed that they play a role in the pathology of DMD. This is emphasized by the lack of the 50 kDa glycoprotein in DMD and severe childhood autosomal recessive muscular dystrophy (SCARMD) (Matsumura *et al.*, 1992a), and a drastic reduction of all these proteins in sporadic myopathy (Matsumura *et al.*, 1993a). There is also an abnormality of the expression of dystrophin-associated proteins in another form of severe muscular dystrophy called Japanese Fukuyama-type (Matsumura *et al.*, 1993b). These dystrophin-associated proteins may prove to be involved in other neuromuscular diseases.

VI. Regulation of Dystrophin Expression

Expression of the DMD gene is developmentally controlled in skeletal muscle. There are no detectable levels of dystrophin expressed until the time of myotube formation (Lev *et al.*, 1987) and/or biochemical differentiation, when muscle-specific genes are expressed (Miranda *et al.*, 1988). The dystrophin gene is therefore expressed similarly to other muscle-specific genes (Endo and Nadal-Ginard, 1987; Chamberlain *et al.*, 1985; Jaynes *et al.*, 1986; Klarsfield *et al.*, 1987).

Several isoforms of dystrophin mRNA have been described. They are believed to be the result of either tissue-specific (Nudel *et al.*, 1989; Feener *et al.*, 1989) or development-specific (Bies *et al.*, 1992b; Clerk *et al.*, 1992) alternative splicing of the large primary transcript. Multiple forms of dystrophin have been detected in brain tissue, smooth, skeletal and cardiac muscle, diaphragm and cardiac Purkinje fibers (Bies *et al.*, 1992b). The DMD gene is regulated developmentally and tissue specifically to produce many potentially functionally distinct isoforms.

VII. Proposed Function of Dystrophin

The varying cellular locations and the number of tissue- and development-specific isoforms of dystrophin suggest that the molecule may play functionally diverse roles. Currently, there are three hypotheses concerning dystrophin function in skeletal muscle (Ahn and Kunkel, 1993). 1) Dystrophin may play a role in membrane stability (Ohlendieck *et al.*, 1991; Ohlendieck and Campbell, 1991a; Watkins *et al.*, 1988); 2) dystrophin may be involved in linking the intracellular contractile force to the extracellular environment (Straub *et al.*, 1992; Porter *et al.*, 1992; Tidball and Law, 1991); and 3) dystrophin may be involved in the functional organization of membrane topology (Peng and Chen, 1992; Torelli *et al.*, 1992) or the maintenance of local concentrations of membrane components (Tidball and

Law, 1991; Ervasti *et al.*, 1990). Though the categories are broad, they are also related, and dystrophin may play more than one of these roles.

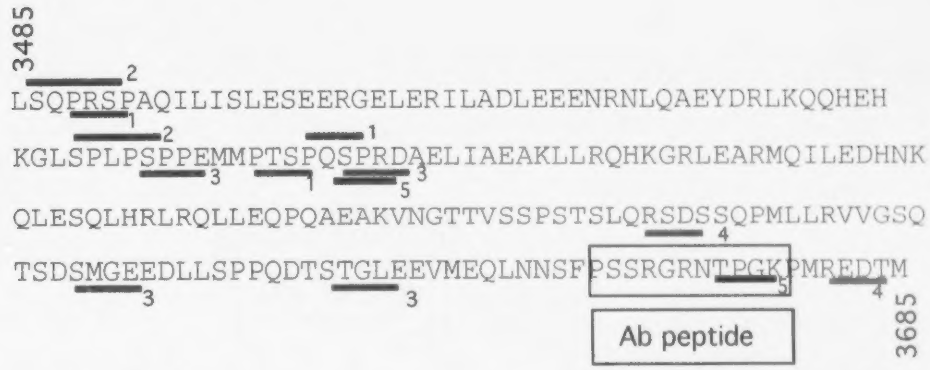
VIII. The Carboxyl-Terminal Domain of Dystrophin

The carboxyl-terminal domain of dystrophin is an important domain to study because it is a fairly distinctive region of the protein. As mentioned earlier (section III), this region is similar to an autosomal homologue DRP (87% amino acid identity to the carboxyl terminus of dystrophin), 70-75 kDa and 116 kDa alternative protein products, and an 87 kDa protein (27% amino acid identity to the carboxyl terminus of dystrophin) cloned from *Torpedo* electric organ. Several facts demonstrate the importance of the dystrophin carboxyl domain. Deletions leading to the loss or alteration of the carboxyl terminus of the protein produce the most severe forms of DMD (Koenig *et al.*, 1989; Bulman *et al.*, 1991), suggesting that this domain of dystrophin must be essential for normal muscle function (Koenig *et al.*, 1989; Bulman, 1991; Bies *et al.*, 1992a). Its structural and functional importance is illustrated by its strong conservation between chicken and human genes (Lemaire *et al.*, 1988)

One clue to the regulation of dystrophin behavior may be its cytoskeletal properties. Cytoskeletal proteins are often involved in protein-protein interactions which are controlled by phosphorylation of specific amino acid residues, for example desmin (homotypic, two of the same proteins interacting) (Geisler and Weber, 1988; Inagaki *et al.*, 1988), vimentin (homotypic) (Inagaki *et al.*, 1987), caldesmon to thin filaments (Yamashiro *et al.*, 1991), caldesmon to actin and calmodulin (Mak *et al.*, 1991a), microtubule-binding activity of microtubule-associated p220 (Shiina *et al.*, 1992), microtubule assembly and disassembly (Gotoh *et al.*, 1991), neurofilaments H and M (Jones and Williams, 1982; Julien and Mushynski, 1982), synapsin 1a to actin (Bähler and Greengard, 1987; Petrucci and Morrow, 1987), and the nuclear lamins (homotypic) (Peter *et al.*, 1991). Analysis of the carboxyl 200 amino acids of dystrophin reveals several potential kinase consensus phosphorylation sites (see figure 1-2). It was discovered in our laboratory that dystrophin is phosphorylated *in vivo* in primary rat skeletal muscle cell culture (Milner *et al.*, 1993), but the kinase(s) responsible for this phosphorylation in skeletal muscle remain to be characterized and/or identified.

Figure 1-2. Amino acid sequence of the carboxyl terminus of dystrophin with identified kinase consensus sites

Potential kinase consensus sequences within the carboxyl 202 amino acids of dystrophin are listed and identified. Slashes indicate that either of the amino acids separated by the slash could substitute for each other. For glycogen synthase kinase, S(P) designates that a serine residue that must first be phosphorylated by another kinase, and S* represents the residue phosphorylatable by glycogen synthase kinase. Kinase sites references are: 1) Clark-Lewis *et al.*, 1991; 2) Fiol *et al.*, 1987; 3) Marin *et al.*, 1986; 4) Payne *et al.*, 1983; 5) Moreno and Nurse, 1990). The box indicates the amino acid sequence of a synthetic peptide used in this study to generate antibodies. These antibodies were then used in the experiment of figure 3-1.


3485
LSQPRSPAQILISLESEERGELERILADLEEENRNLQAEYDRLKQQHEH
KGLSPLPSPPEMMPTSPQSPRDAELIAEAKLLRQHKGRLEARMQILEDHNK
QLESQLHRLRQLLEQPQAEAKVNGTTVSSPSTSLQRSDSSQPMLLRVVGSQ
TSDSMGEEDLLSPPQDTSTGLEEVMEQLNNSFPSSRGRNTPGKPMREDTM
1
2
3
4
5
Ab peptide
3685

	Kinase	Consensus Sequence
—1	Mitogen Activated Protein Kinase -p42/p44	PXS/TP
—2	Glycogen Synthase Kinase 3	S*XXXS(P)
—3	Casein Kinase 2	S/TXXD/E
—4	Calmodulin kinase	RXXS/T
—5	cdc2 kinase -p34	XS/TPXK/R

It was the goal of this research project to identify the dystrophin protein as a substrate of kinase activity(s) present in soluble skeletal muscle extracts, and to characterize the activity(s) with the potential of further understanding the significance of dystrophin phosphorylation.

My study of dystrophin as a substrate for phosphorylation involved analysis of kinase activity from soluble extracts prepared from rat and rabbit skeletal muscle. Such extracts contain phosphatases as well as other regulatory kinases or proteins, and their use may provide information on complex regulatory systems relevant to dystrophin. This study investigated the phosphorylation of dystrophin by soluble skeletal muscle extracts, purified casein kinase II (CK II) and mitogen activated protein kinase (MAP kinase). CKII was included because of the presence of consensus sequences in the carboxyl terminus of dystrophin, and the possibility that it may phosphorylate dystrophin in a structural manner (see section IX, this chapter). MAP kinase was included also because of the presence of its consensus sequences in the carboxyl terminus of dystrophin, and because MAP kinase is known to control the behavior of other cytoskeletal proteins (see section X, this chapter).

IX. Casein Kinase II

It is possible that dystrophin is subject to a structural type of phosphorylation, where the protein is phosphorylated early on in its life-span, and the phosphate is not removed. Casein kinases are candidates for this type of phosphorylation (Hemmings *et al.*, 1982; Safer and Jagus, 1979). The carboxyl terminus of dystrophin contains consensus sites for casein kinases.

The casein kinases are ubiquitous kinases of unknown biological function. It has been suggested that CK II may have a role in cell proliferation and growth (Ackerman *et al.*, 1988; Carroll and Marshak 1989; Sommercorn *et al.*, 1987; Ackerman *et al.*, 1990 Filhol *et al.*, 1990; Filhol *et al* 1991). CK II is an acidophilic Ser/Thr protein kinase and recognizes carboxylic determinants (Glu or Asp) (Pinna *et al.*, 1984) or phosphorylated residues (Meggio *et al.*, 1988; Litchfield, *et al.*, 1990). This is unlike most Ser/Thr protein kinases which prefer basic residues as specificity determinants. The *in vivo* or physiological substrates of CK II include SV40 large T antigen (Grasser *et al.*, 1988; Scheidtmann *et al.*, 1982), glycogen synthase (Poulter *et al.*, 1988; Cohen *et al.*, 1982), phosphatase inhibitor 2 (Holmes *et al.*, 1986), heat shock protein 90 (Lees-Miller and Anderson, 1989), cAMP-dependent protein kinase R_{II} (Hemmings *et al.*, 1982; Carmichael *et al.*, 1982), low density lipoprotein receptor (Kishimoto *et al.*, 1987), acetyl CoA carboxylase (Haystead *et al.*, 1988), troponin T (Pinna *et al.*, 1981).

X. Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAP kinases) are protein serine/threonine kinases that are activated by both tyrosine and threonine phosphorylation (Anderson *et al.*, 1990). MAP kinases can be activated by mitogenic (Mutoh *et al.*, 1988; Hoshi et al, 1988; Miyasaka *et al.*, 1990; Ahn *et al.*, 1990; Yoshimasa et al, 1992; Sanghera et al, 1990) and non-mitogenic signals (Ray and Sturgill, 1987; Gomez *et al.*, 1990; Lloyd and Wooten, 1992; Ohmichi et al, 1992). MAP kinase substrates are diverse and include S6 kinase-II (Sturgill *et al.*, 1988), myelin basic protein (Pelech et al., 1988; Circirelli *et al.*, 1988), caldesmon (Childs *et al.*, 1992), transcription factors c-*jun* (Pulverer *et al.*, 1991), c-*myc*, (Alvarez *et al.*, 1991) and $p62^{TCF}$ (Gille *et al.*, 1992), MAP kinase activated protein kinase-2 (Stokoe *et al.*, 1992) and the microtubule associated protein tau (Drewes *et al.*, 1992).

OBJECTIVES

Dystrophin is believed to be a cytoskeletal protein which may stabilize and/or organize the membrane or membrane components, and/or mediate interactions between the extracellular matrix and the actin-cytoskeleton. Accordingly, it follows that dystrophin's behavior may be regulated in ways similar to those of other cytoskeletal proteins and/or other proteins involved in intracellular signalling. Dystrophin is phosphorylated *in vivo* in cultured primary rat skeletal muscle cells (Milner *et al.*, 1993), but the responsible kinases remain to be identified. The specific objectives of this research project were:

1) to identify the carboxyl terminus of the dystrophin protein as a substrate of a kinase(s) activity present in soluble skeletal muscle extracts,

2) to determine if the phosphorylation of the carboxyl terminus of dystrophin is multisite,

3) to partially characterize and possibly identify the responsible kinase(s) present in soluble skeletal muscle extracts,

4) to investigate the relevance of some of the kinase consensus sites in dystrophin's carboxyl terminus by employing purified kinase(s).

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CHAPTER 2 - METHODS

I. Materials

γ[^{32}P]ATP (3000 Curies/mmol) was purchased from New England Nuclear, Boston, Massachusetts. Unlabeled ATP, Triton X-100, MOPS, DTT, PMSF, benzamidine, β-glycerophosphate and protease inhibitors were from Sigma, St. Louis, Missouri. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and molecular weight markers were from Bio-Rad, Hercules, California. Standards were Bio-Rad low range molecular weight marker proteins: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). Immobilon (polyvinylidene difluoride was from Millipore, Mississauga, Ontario. Phosphoamino acid standards (figure 2-3), phosphatase inhibitor-2, okadaic acid and microcystin LR were generous gifts from Dr. C. Holmes of the University of Alberta, Edmonton, Alberta. Cell lines Sol8 and L6 were generous gifts from Dr. P. Holland of the Neurological Institute in Montreal, Quebec. Canine pancreatic and recombinant calreticulin, and rabbit skeletal and canine cardiac calsequestrin were gifts from Shairaz Baksh of the University of Alberta, Edmonton, Alberta. X-ray film and the thin layer cellulose plates were from Kodak at Rochester, New York. MAP kinase, p44mpk, and anti-erk1 antibodies were gifts from Dr. S. Pelech of the University of British Columbia and Kinetek Biotechnology Corporation at Vancouver, British Columbia. Cyanogen bromide-activated Sepharose, DEAE-Sepharose CL6B, Phenyl-Sepharose, IgG-Sepharose 6FF, Mono-Q and glutathione sepharose 4B,pGEX-3X, pRIT2T, and *Escherichia coli* strain N4830-1 were from Pharmacia, Baie d'Urfe, Quebec. The *E. coli* strain BNN103 was a gift from Dr. J. Weiner of the University of Alberta, Edmonton, Alberta. Heparin Sepharose, polylysine-agarose and phosphocellulose were from Sigma. DEAE-cellulose was from Whatman Inc. Hydroxylapatite was from Bio-Rad. Ammonium sulphate was from BDH. IPTG and myelin basic protein were from Sigma. Ninhydrin was from Chromatographic Specialty, Brockville, Ontario. All other chemicals used were of the highest grade commercially available.

II. SDS-Poly-Acrylamide Gel Electrophoresis (PAGE) and Immunoblotting

Samples were mixed with Laemmli sample buffer (2x concentration, 20% glycerol, 2% β-mercaptoethanol, 4% SDS, 0.130 M Tris pH 6.8, 1% Bromophenol Blue) and SDS-PAGE was carried out using 10% or 12.5% polyacrylamide gels (Laemmli, 1970). After electrophoresis, the proteins were stained with Coomassie blue. Gels were incubated for 30 min in coomassie blue stain (0.5 g of R250 Coomassie brilliant blue, 25% 2-propanol, 10% acetic acid in distilled water), and destained overnight in a destain solution (7.5% acetic acid and 10% methanol in distilled water). Alternatively, proteins were transferred to nitrocellulose (Towbin *et al.*, 1979) for immunoblotting or to Immobilon membrane (Matsudaira, 1987) for phosphoamino acid analysis.

Polyacrylamide gels were dried on Whatmann 3MM filter paper under vacuum in a Bromma slab gel dryer. Autoradiography of labelled proteins in dry polyacrylamide gels was carried out with an intensifying screen and Kodak X-ray film in a Dupont cassette at -70°C.

Immunoblotting for the experiment of figure 3-1 was carried out as described by Michalak *et al.*, (1990). Nitrocellulose membranes were soaked in phosphate-buffered saline (PBS: 0.137 M NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.2) and its non-specific sites blocked with 5% milk powder in PBS for 15 min at room temperature. The primary antibody (rabbit polyclonal, section IV) was added at a dilution of 1:500 in 1% milk powder and incubated at room temperature for 1 hour. The membranes were washed for 15 min twice in PBS plus 0.05% Tween-20, and once in PBS. The secondary antibody (goat anti-rabbit antibody with a hydrogen peroxidase conjugate) was added at a dilution of 1:2000 in 1% milk powder and incubated at room temperature for 1 hour. The membranes were washed as described above. The detection system employed goat anti-rabbit secondary antibodies linked to hydrogen peroxidase with 0.3% 4-chloro-1-napthol and 0.12% hydrogen peroxide for color development.

Immunoblotting for the experiment of figure 4-4 was carried out according to Kinetek (manufacturer) instructions. Basically membranes were blocked in 5% milk powder with Tris-buffered saline (0.137 M NaCl, 2.68 mM KCl, 0.025 M Tris-HCl, pH 7.4) after which the primary anti-erk1 antibody was added to a dilution of 1:500 in 1% milk powder and 0.05% Tween-20 in Tris-buffered saline. The detection method was alkaline phosphatase-coupled anti-rabbit antibody using bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color development.

III. Construction of the plasmids used to generate fusion proteins

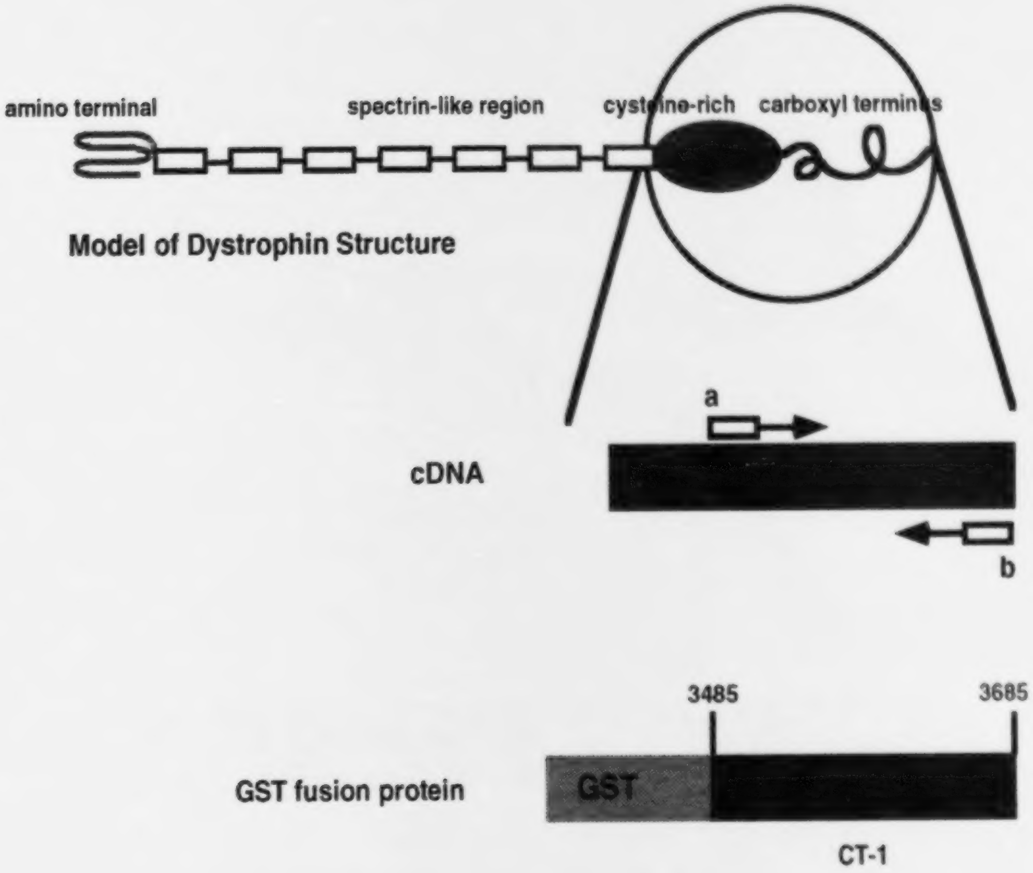
The polymerase chain reaction was used to synthesize the cDNA encoding the carboxyl 201 amino acids of dystrophin. The template was a human cDNA clone spanning the region 7800 base pairs from the 5′ end of the dystrophin cDNA, cDMD 9-14 (American Type Culture Collection, depositor Dr. L. M. Kunkel). Its nucleotide sequence is described by Koenig (*et al.*, 1987). The 5′ forward primer used to generate CT-1, primer a (see figure 2-1), corresponds to nucleotides 10661-10681 of the dystrophin cDNA, and encodes amino acids 3485-3491 of the protein. The 3′ reverse primer used to generate CT-1, primer b (see figure 2-1), corresponds to nucleotides 11294-11317 of the dystrophin cDNA, which are in the non-coding region. The polymerase chain reactions were performed in a buffer which contained 50 mM KCl, 10 mM Tris pH 8.3, 0.01% gelatin, 200 μM of each dNTP, 1.5 mM MgCl, 2.5 units of Taq I polymerase, 50 ng of the DNA template and 200 ng of each of the primers. The products were purified by PAGE and cut with EcoR1 restriction endonuclease. Production of the fragments and subcloning was performed by Dr. R. E. Milner of Dr. Michalak's laboratory.

IV. Expression and Purification of the Carboxyl Terminus of Dystrophin as a fusion protein

The last 201 amino acids at the carboxyl terminus of dystrophin were expressed as a glutathione-S-transferase (GST) or as a protein A (PA) fusion protein, and were designated respectively as GST-CT-1 or PA-CT-1 (Milner *et al.*, 1992) (figure 2-1). Briefly, DNA encoding the carboxyl 201 amino acids of dystrophin was subcloned into a pGEX-3X plasmid behind sequences coding for GST, or into plasmid pRIT2T behind sequences coding for protein A. The CT-1 nucleotide sequence was confirmed by Taq polymerase cycle DNA sequencing using an automated Model 373A DNA sequencer from Applied Biosystems (Mississauga, Ontario) at the University of Alberta DNA Sequencing Laboratory, Edmonton, Alberta. The pGEX-3X and pRIT2T

Figure 2-1. Construction of the dystrophin fusion protein GST-CT-1

The portion of the dystrophin protein which corresponds to CT-1, dystrophin amino acids 3485-3685, was produced as a fusion protein with glutathione-S-transferase (GST) amino acid sequences preceding it. The coding DNA sequences for CT-1 were prepared for expression by polymerase chain reaction from a human dystrophin cDNA template (cDMD 9-14), using primers a (5′-TGGAGTTCGCTGAGCCAGCCTCGTAGTCCT-3′) and b (5′-TGGAATTCACTAAGGACTCCATCGCTCTGC-3′), designated here with boxes and arrows.



Protein Fragment:

1. Glutathione-S-Transferase fusion protein called GST-CT-1 produced in pGEX-3X

plasmids were transformed into *Escherichia coli* cell strains BNN103 and N4830-1 respectively.

BNN103 cells were transformed by incubating 20 ng of DNA with the cells on ice for 30 min in a buffer containing 100 mM KCl, 45 mM MnCl, 10 mM $CaCl_2$, and 10 mM K-MES (2-[N-Morpholino]ethanesulfonic acid) pH 6.3, followed by a 40 sec heat shock at 42 ^oC. Four volumes of SOC media (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added, and the cells incubated for 1 hour at 37 ^oC with shaking. The cells are then plated on Luria-Bertani plates (media containing 1% agar) with ampicillan (50 µg/mL), and incubated overnight at 37 ^oC.

N4830-1 cells were transformed according to the procedure provided by Pharmacia. Basically, the cells were incubated on ice for 30 min with 20 ng of DNA, in a buffer containing 100 mM KCl, 45 mM MnCl, 10 mM $CaCl_2$, 3 mM $HACoCl_3$ and 10 mM K-MES pH 6.3. The cells were subjected to a heat shock of 42 ^oC for 45 sec, iced and placed in SOC media, which was followed by incubation at 30 ^oC for 1 hour. The cells are then plated on Luria-Bertani plates (media containing 1% agar) with ampicillan (50 µg/mL), and incubated overnight at 37 ^oC.

The GST fusion proteins were produced in BNN103 cells that were induced with 0.1 M isopropyl β-D-thiogalactopyranoside (IPTG) at log-phase (A_{600} 0.6-1.0), growing at 37 ^oC in Luria-Bertani medium (1.0% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v) and 1.0% NaCl (w/v), pH 7.0) containing ampicillan (50 µg/mL). Four hours after induction , the cells were centrifuged at 3500 x g for 15 min at 4 ^oC, the pellet resuspended in PBS containing 0.1% Triton X-100 and the cells lysed using a French press. The lysates were centrifuged at 100 000 x g for 45 min at 4 ^oC and processed by glutathione-Sepharose 4B affinity chromatography (column size 2 x 12 cm) in PBS plus 0.1% Triton X-100. GST-containing proteins were eluted with a buffer containing 5 mM glutathione and 50 mM Tris pH 8.0, dialysed against 50 mM Tris pH 7.1, containing 1 mM EDTA and subjected to MonoQ FPLC. The samples were loaded onto the column equilibrated with 50 mM Tris pH 7.1 and 1 mM EDTA, and washed with the same buffer. The proteins were eluted with a linear salt gradient (0.0-0.75 M NaCl). Positive fractions were identified based upon comparison of fractions processed from strains overexpressing the fusion protein, with fractions processed from strains not overexpressing the fusion protein.

The protein A fusion proteins were expressed in N4830-1 cells, which contain a temperature-sensitive λ cl_{857} repressor. Cells at early stationary phase (A_{600} 0.8-2.0) at 30 °C were rapidly shifted to 42 °C and incubated for 90 min. The cells were centrifuged at 5000 x g for 5 min and the pellet resuspended in a buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20, and lysed in a French press as described above. The lysate was then centrifuged at 100 000 x g for 45 min at 4 °C. The pellet was resuspended in a buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20. Protein A-containing proteins were loaded onto an IgG-Sepharose 6FF affinity chromatography colum (2 x 15 cm), and eluted with a buffer containg 0.5 M acetic acid pH 3.4, after a wash of 2 bed volumes of 5 mM acetic acid pH 5.0. Positive fractions were identified based upon comparison of fractions processed from strains overexpressing the fusion protein, with fractions processed from strains not overexpressing the fusion protein.

The identity of the fusion proteins were confirmed immunologically by anti-dystrophin (synthetic peptide) antibody, anti-PA-CT-1 antibody and anti-GST antibody reactivity (see section V this chapter). GST-CT-1 was present either as a full length fusion protein, or as a full length fusion protein and a truncated fusion protein product. The reason for the difference was not investigated, but the the proteins are from different preparations.

V. Production of Antibodies

Rabbit polyclonal antibodies were made against the protein A, GST dystrophin fusion proteins, PA-CT-1 and GST-CT-1, and purified GST. Rabbit polyclonal antibodies were also made against a synthetic peptide which consisted of amino acids 3668-3679 of the carboxyl terminus of dystrophin (NH2-P-S-S-R-G-N-T-P-G-K-P-COOH), synthesized at the Alberta Peptide Institute as a keyhole limpet haemocyanin conjugate (Milner *et al.*, 1992) (figure 1-1). Anti-erk1-CT antibodies were also polyclonal, produced in New Zealand White rabbits. The immunogen in this case was a 38 residue peptide (amino acid sequence NH_2-P-F-T-D-M-E-L-D-D-L-P-K-E-R-L-K-E-R-L-K-E-L-I-F-Q-E-T-A-R-F-Q-P-G-A-P-E-A-P-COOH) based upon the carboxyl residues 333-367 of the rat 43 kDa erk1 map kinase. The peptide was coupled to keyhole limpet haemocyanin prior to immunization.

The rabbit polyclonal antibodies made against dystrophin fusion proteins or the dystrophin synthetic peptide were produced by subcutaneous injection of 1.0 mg of the respective protein emulsified in Freund's complete adjuvant into New Zealand White rabbits. After 2 weeks the immunization was repeated with 1.0 mg of the protein emulsified in Freund's incomplete adjuvant. Sera were screened 2-3 days later against purified proteins and crude dystrophin preparations that had been transferred to nitrocellulose. Serum containing the anti-dystrophin (synthetic peptide) antibody was affinity purified on a column of bovine serum albumin-coupled dystrophin peptide coupled to CNBr-activated sepharose. The purified anti-dystrophin (synthetic peptide) antibodies recognized the full length GST-CT-1 protein, PA-CT-1, native dystrophin and not GST or protein A. Serum containing the anti-PA-CT-1 antibody was affinity purified by sequential and repeated incubations with blots carrying GST-CT-1 in 1% milk powder made in PBS. The antibodies were eluted with 0.2 M glycine pH 2.0, and neutralized immediately by the addition of 1 M Tris to pH 7.0. Anti-PA-CT-1 antibodies recognized purified PA-CT-1, full length and truncated GST-CT-1, native dystrophin and not GST nor protein A. Serum containing anti-GST-CT-1 antibodies was affinity purified by sequential and repeated incubations with blots carrying PA-CT-1 in 1% milk powder made in PBS. The antibodies were eluted with 0.2 M glycine pH 2.0, and neutralized immediately by the addition of 1 M Tris to pH 7.0. Anti-GST-CT-1 antibodies recognized purified full length and truncated GST-CT-1, PA-CT-1, native dystrophin, but not GST nor protein A. Anti-GST antibody recognized purified GST, and purified full length and truncated GST-CT-1, but not PA-CT-1 nor native dystrophin.

The polyclonal rabbit anti-erk1-CT antibodies were produced by Kinetek in New Zealand White rabbits which were subcutaneously injected with the synthetic peptide every 4 weeks (Kinetek Biothechnology Corporation). The sera collected from the animals was applied to an agarose column which was thio-linked to the peptide. The antibody was eluted with

0.1 M glycine pH 2.5, and the solution neutralized to pH 7.0 by the addition of saturated Tris. The antibodies recognized the 43 kDa Map kinase encoded by the erk1 gene 42 kDa MAP kinase encoded by the erk2 gene, and the 44 kDa MAP kinase encoded by the mpk gene. It also appeared to recognize other putative MAP kinases in the range of 40 to 70 kDa. It recognizes various MAP kinases in cytosolic and nuclear extracts from sea star, clam, frog, chicken, mouse, rat sheep and human.

VI. Preparation of Soluble Skeletal Muscle and Liver Extracts

New Zealand White rabbits were sacrificed by the injection of euthanyl intravenously into the marginal ear vein. Rabbit and rat skeletal muscle groups visually identified as red (slow- twitch) were immediately excised, minced and homogenized for 30 seconds at setting 5 using a Polytron homogenizer, in 2.5 volumes (v/w) of extraction buffer which consisted of 10 mM dibasic sodium phosphate pH 7.5, 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, in the presence or absence of 1 μM okadaic acid, 10 mM PMSF, 1mM benzamidine, and a 1 in 2000 dilution of a protease inhibitor cocktail in 5% DMSO consisting of 0.5 μg of *N*-[*N*-(L-3-trans-carboxyoxirane-2-carboxyl)-L-leucyl]-agmatine/mL, 0.1 μg aprotinin/mL, 0.5 μg leupeptin/mL, 0.5 μg pepstatin.mL, 50 μg L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, 0.1 μg of (4-amidinophenyl)-methansulfonyl fluoride/mL, 50 μg *N*-(L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L -tryptophan/mL (Michalak *et al.*, 1990). The homogenate was centrifuged at 6000 x g for one hour at 4°C. The resulting supernatant was filtered through glass wool, and centrifuged at 100 000 x g for one hour at 4°C. The supernatant was treated in one of three ways: 1) stored in 50% glycerol, 2) fractionated consecutively into 25% (144 g/L), 45% (125 g/L) and 65% (134 g/L) saturated ammonium sulfate, centrifuged at 27, 000 x g, and dialyzed overnight at 4 °C in two changes each of 4 L (1/160 mL) of Buffer A (which consisted of 50 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM PMSF, 1 mM benzamidine and the cocktail (see above) of protease inhibitors) plus 2 mM Ca^{++} or Buffer B (which consisted of 12.5 mM MOPS pH 7.2, 7.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM DTT, 0.5 mM EGTA, 0.05 mM NaF, 1 mM PMSF, 1 mM benzamidine and the cocktail (see above) of protease inhibitors) or 3) immediately dialyzed in Buffer A or Buffer B without ammonium sulfate fractionation. These crude kinase preparations were stored in 50% glycerol at -20 °C. The identical procedure was used for the preparation of soluble rabbit liver extracts.

VII. Fractionation of the Soluble Muscle Extract for MAP Kinase Detection

The soluble muscle extract made in the presence of okadaic acid was fractionated in Dr. S. Pelech's laboratory by FPLC chromatography using a Mono Q 10/10 column. The extract was applied to the column, and proteins that bound were eluted with a 0.0-0.8 M linear NaCl gradient in a buffer containing 10 mM MOPS pH 7.2, 2 mM EDTA, 1 mM DTT. The collected fractions were analyzed for immunoreactivity with rabbit polyclonal antibodies against MAP kinase(s) (Childs *et al.*, 1992), and for phosphotransferase activity against GST-CT-1 and myelin basic protein.

VIII. Phosphorylation Reactions

Preweighed amounts of dessicated unlabeled ATP were purchased from Sigma, and dissolved in water to a calculated concentration. This was then diluted with water to 500 µM, with the addition of $\gamma[^{32}P]$ ATP (1:500). This mixture was then diluted to 100 µM in each reaction, and aliquots counted in scintillant to determine the activity required to calculate stoichiometry.

Soluble muscle extracts (in either Buffer A or Buffer B) were employed as crude kinase preparations at a protein concentration of 2.5 mg/mL. Substrate was added at 0.25 mg/mL. Any "prephosphorylation" incubations were performed at room temperature for 10 min. Phosphorylation of substrates with CKII was performed using a buffer containing 50 mM Tris pH 7.4, 150 mM KCl, 10 mM MgAcetate. Phosphorylation of substrates with MAP kinase was carried out in Buffer B in the presence 0.5 mg/mL substrate. All of the phosphorylation reactions were initiated by the addition 100 µM $\gamma[^{32}P]$ATP, and carried out at 30 °C. The reactions for autoradiograph analysis were terminated by the addition of sample buffer and separated on SDS-PAGE. The proteins were then stained with Coomassie blue. Gels were dried and exposed to X-ray film.

Incorporation of ^{32}P into GST-CT-1 for MAP kinase data was determined by trichloroacetic acid precipitation of proteins on Whatman 3MM paper (Ahmad *et al.*, 1982). Strips of 3MM paper were soaked in 5% trichloroactetic acid and 0.25% sodium tungstate and dried. Reactions of 50-100 µL volumes were spotted onto pieces of the pretreated 3MM. After the pieces were dry, they were washed 3 times for 15 min each in 5% trichloroacetic acid and 0.25% sodium tungstate. The pieces were then rinsed in acetone and allowed to dry. The individual pieces of paper were then placed in scintillant and the amount of ^{32}P counted.

IX. Phosphoamino Acid and Phosphopeptide Analysis

Two different methods of phosphoamino acid analysis were employed. Phosphoamino acid analysis for figure 3-3c was carried out according to Kamps (1991). Phosphorylated proteins were separated by SDS-PAGE, and transferred to Immobilon membranes. The appropriate bands were excised and subjected to hydrolysis in 6N constant boiling HCl at 110 °C for 1 hour after degasing. The supernatant was collected and lyophilized, the resultant pellet resuspended in water. Samples were subjected to one dimensional electrophoresis in pH 1.8 electrophoresis buffer (water:acetic acid:formic acid, 900:80:20) on Whatman 1M paper at 3000 V for 120 minutes, resolving phosphoserine from phosphothreonine and phosphotyrosine. Phosphoamino acid standards were stained with ninhydrin. Phosphoamino acids labeled with ^{32}P were visualized by autoradiography.

Phosphoamino acid analysis and phosphopeptide mapping for MAP kinase data (figure 4-3) were performed as described by Sanghera *et al.*, (1991). For phosphoamino acid analysis, phosphorylated GST-CT-1 was placed in 6N constant boiling HCl, degased and hydrolysed at 110 °C for 2 hours. The sample was diluted with double deionized H_2O, lyophilized and resuspended in pH 1.8 electrophoresis buffer. The hydrolyzed sample was subjected to electrophoresis on thin layer cellulose at 400 V for 2 hours and then autoradiographed. Tryptic digestions for phosphopeptide mapping were carried out with phosphorylated GST-CT-1 and trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated from Sigma) (50:1 weight ratio) at 37 °C for 20 hours in 50 mM $NH4C0_3$ pH 8.0. The digested samples were lyophilized, suspended in pH 1.8 thin layer electrophoresis buffer, and subjected to electrophoresis on thin layer cellulose plates at 400 V for 2 hours. The second dimension consisted of ascending chromatography in 1-butanol:pyridine:acetic acid:water (40:50:10:40).

X. Production and Purification of Recombinant p13

p13 is a protein product of the yeast suc1 gene. The plasmid construct (a gift from Dr. T. Hunt, Cambridge University) used for producing recombinant p13 was produced in *E. coli* (BL21), induced by the addition of IPTG, and purified according to a modified procedure of Mak (*et al.*, 1991). The cells from 1.6 L of medium were harvested by centrifugation at 3000 x g and resuspended in 35 mL of 50 mM Tris pH 7.0, 1 mM EDTA and the protease inhibitor cocktail (see above). The cell suspensions were then lysed in a French press and the resulting insoluble particles and membranes were pelleted at 100, 000 x g at 4 ^{o}C. The supernatants were frozen in liquid nitrogen and stored at -20 ^{o}C until further use. The p13 was purified first by size exclusion chromatography. Eight mL of the 100, 000 x g supernatant was loaded onto 1 L of DEAE-Sepharose CL6B in the 50 mm Tris pH 7.0 and 1 mM EDTA (flow rate of 15 mL per hour). The protein was eluted with 250 mL of the same buffer, and 3 mL fractions were collected. Fractions containing p13 were identified, after SDS-PAGE and Coomassie blue staining, by comparisons with lysates which did not express p13. These fractions were pooled and concentrated in an Amicon concentrator (YM10 membrane). The CL6B Sephadex samples of p13 were then loaded onto a large Mono-Q FPLC column (10/10) and collected in the column wash (50 mM Tris pH 7.0 and 1 mM EDTA). The fractions containing large amounts of pure p13 were pooled and concentrated, followed by coupling of p13 to CNBr-activated Sepharose 4B as per the manufacturer's instructions.

XI. Affinity Chromatography of Soluble Skeletal Muscle Extracts on p13-Sepharose

One mL aliquots of soluble muscle extract dialyzed in Buffer A plus 2 mM Ca^{++} were preincubated, each with 100 µL of a 50% slurry of a control (ethanolamine blocked) CNBr-activated Sepharose 4B. The samples were centrifuged briefly at 6000 x g and the pellets discarded. The supernatants were added to 60 µL of a 50% slurry of p13-affinity-Sepharose, and the samples incubated overnight (15 hours) at 4 ^{o}C with constant rocking.

The samples were centrifuged briefly as above, and the pellets washed with centrifugation 10 times with 1 mL of Buffer A containing 2 mM Ca^{++}. Upon the last wash, the pellets were transferred to a new tube. Bound proteins were then analysed by SDS-PAGE followed by silver staining, or by their ability to phosphorylate GST-CT-1.

The phosphorylation reaction contained 30 µL of a 50% slurry of p13-affinity Sepharose, Buffer A plus 2 mM Ca^{++}, 0.25 mg/mL GST-CT-1, 100 µM $\gamma[^{32}P]$ATP. The reactions were started by the addition of $\gamma[^{32}P]$ATP and incubated for 120 min at 30 °C. They were terminated by the addition of sample buffer. The proteins were separated by SDS-PAGE and subjected to autoradiography.

XII. Preparation and Maintenance of Primary and Cell Line Culture

L6 and Sol8, rat skeletal muscle cell lines, (both gifts of Dr. P. Holland, Neurological Institute, Montreal, Quebec) were subcultured in Dulbecco's modified Eagle's medium (DMEM) and 10% horse serum, and incubated at 37 °C with 5% CO_2. The cells were subcultured every three days using 0.25% trypsin for 3 min at 37 °C to remove the cells from the plates. Cells were collected, washed by centrifugation at 1000 x g for 4 min at room temperature in fresh medium (DMEM plus 10% horse serum), and counted using a Coulter counter. For the experiments, the cells were grown in DMEM and 5% horse serum, and the media changed every three days. L6 cells do not express dystrophin (Lev *et al.*, 1987). Both Sol8 and the primary rat skeletal muscle cells were maintained on gelatinized plates. Gelatinized plates were prepared by incubating plates containing 3 mL of 0.1% gelatin in sterile water overnight at 4 °C, decanting the gelatin and allowing the plates to dry. Cells were plated at a density of 3×10^6-5×10^6 cells per plate at day 0, and were allowed to fuse. Cells were harvested on each day following initial seeding for 7 (L6) or 9 (Sol8) days (see this chapter section XIII). The cells were tested for the presence of mycoplasma by a Gen-Probe mycoplasma rapid detection system (Gen-Probe Inc., Sandiego, California).

Fusion of the myoblasts into mytotubes was identified by visualization of multinucleated cells after staining of the fixed cells. The cells were fixed using 3% glutaraldehyde in PBS for 3 min, and washed with distilled water. The cells were then stained by incubating in hematoxylin for 3 min and washed in tap water, then stained with eosin for 5 min, followed by a wash in distilled water and storage in glycerol.

Primary cultures were started from the hind thigh and calf muscles of 1-3 day old rat pups. The tissue was minced and placed into a trypsinization flask with 10 mL of 0.3% trypsin. The flask was then placed in the incubator at 37 °C and 5% CO_2 with stirring. When the solution became cloudy, it was decanted into a 15 mL conical tube, avoiding large particulate matter. The flask was refilled to 10 mL and replaced in the incubator for further digestion. The 15 mL conical tube was filled with an equal volume of DMEM and 10% horse serum and centrifuged at 1000 x g for 4 min at room temperature. The supernatant was discarded and the pellet resuspended in 3-5 mL of DMEM and 10% horse serum and left at room temperature. The trypsinization procedure was continued until stringy lumps were left in the flask. The contents of the tubes were pooled and poured into a syringe, and then filtered through a 75 µm sterile filter (Millipore), followed by a 10 µm sterile filter (Millipore). The cells were plated at 3.0×10^6-5.0×10^6 cells per plate at day 0, in DMEM plus 10% horse serum and 0.5% chick embryo extract, plus 50 µg gentamycin sulfate/penicillin and streptomycin. The cells were allowed to fuse. Cells were harvested on each day following initial seeding for 8 days (see this chapter section XIII).

XIII. Preparation of Cell Culture Extracts

Soluble primary and cell culture extracts employed as crude kinase preparations were made by scraping cells from two (100 mm x 20 mm) culture dishes with a rubber policeman in 0.5 mL of extraction buffer which consisted of 10 mM dibasic sodium phosphate pH 7.5, 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 10 mM PMSF, 1 mM benzamidine, and the cocktail of protease inhibitors. The lysate was homogenized on ice with 30 downward strokes of a Dounce homogenizer, and centrifuged for 5 min in an Eppendorf centrifuge at 12, 000 rpm, at 4 °C. The resulting supernatant was then stored in 50% glycerol at -20 °C. Each extract from each day was prepared in the same volume of buffer. Protein assays (Lowry) were employed to normalize the amount of extract used in subsequent phosphorylation reactions.

XIV. Isolation and Purification of Casein Kinase II

For the isolation of CKII, 2 kg of bovine brain (from Gainers, Edmonton, Alberta) was minced and homogenized using a polytron for 30 seconds at setting five in 4 volumes of isolation buffer which consisted of 10 mM triethanolamine pH 7.4, 5 mM EDTA, 2 mM EGTA, 0.02% sodium azide, 5 mM DTT, 1 mM benzamidine, 1 mM PMSF and the cocktail of protease inhibitors. The homogenate was then centrifuged at 27, 000 x g for one hour at 4 °C. After adjusting the pH of the supernatant to 7.4, it was stirred with 0.5 L of equilibrated DEAE Sephadex-A50 for one hour at 4 °C. The Sephadex was washed 2 times with 4.5 L of triethanolamine isolation buffer, and placed in a large chromatography column. The proteins were eluted using a 1.8 L gradient of 0.0-0.5 M KCl. The fractions (5.5 mL) were assayed by phosphorylation reactions with casein in 50 mM Tris pH 7.4, 150 mM KCl, 10 mM MgAcetate, 100 μM $\gamma[^{32}P]$ ATP. The reactions were started with the addition of ATP and terminated by the addition of sample buffer. The samples were then analyzed by SDS-PAGE and autoradiography.

The active fractions (eluted at 380 mM KCl) were pooled and the proteins therein were precipitated with 55% ammonium sulfate in the isolation buffer. The precipitate was centrifuged at 20, 000 x g for 30 min at 4 °C and resuspended in 50 mL of isolation buffere buffer, and dialyzed overnight. The sample was then loaded onto a 25 mL heparin-Sepharose column, and eluted with a 500 mL gradient of 0.0-1.25 M KCl (6.8 mL fractions) in isolation buffer.

Active samples (460 mM KCl) (determined as above), were dialyzed in isolation buffer, and loaded onto a FPLC MonoQ column (type HR 5/5), and bound proteins were eluted (1 mL/ fraction) with a 100 mL gradient of 0.0-1.0 M KCl. Active fractions (250 mM) were dialyzed against a buffer containing 25 mM potassium phosphate pH 7.4, 5 mM DTT, 1 mM PMSF, 1 mM benzamidine, and protease inhibitor cocktail, loaded onto a 50 mL hydroxylapatite column, eluted with a 100 mL gradient of 25-500 mM potassium phosphate. Active fractions (190 mM potassium phosphate) were pooled, concentrated, and stored in 50% glycerol at -20 °C.

The fractions of each purification step were tested for their ability to phosphorylate casein, and the pooled samples at each step were tested for their ability to phosphorylate canine cardiac calsequestrin. Samples were also tested for their sensitivity to heparin (2 µg/mL). The purified CKII was able to phosphorylate several known CKII substrates including casein, cardiac and skeletal calsequestrin and phosphatase inhibitor-2. The purified enzyme was also inhibited in the presence of heparin (2 µg/mL).

XV. Purification of Mitogen Activated Protein Kinase

MAP kinase was purified in Dr. S. Pelech's laboratory from sea star oocytes as previously reported (Sanghera *et al.*, 1990). Purification to homogeneity was carried out by sequential chromatography on DEAE-cellulose, hydroxylapatite, phosphocellulose, phenyl-Sepharose, heparin-Sepharose, polylysine-agarose, and Mono-Q. Sea stars were collected from beaches in the Vancouver area. Briefly, cytosol was prepared from sea star oocytes arrested in the G2/M phase prior to mitosis, by homogenization in a buffer containing 125 mM β-glycerophosphate, 12 mM EDTA, 2 mM sodium vanadate, 1 mM DTT, 0,5 mM PMSF, pH 7.2 and homogenization at 100 000 x g for 30 min. The cytosol was then diluted 33 fold with a buffer containing 5 mM MOPS pH 7.2, 5 mM EGTA, 5 mM NaF, 1 mM sodium vanadate and 0.25 mM DTT, added to 170 mL (per 4 L diluted cytosol) DEAE-cellulose and stirred gently for 1.5 hours at 4 °C. The DEAE-cellulose was allowed to settle, the supernatant removed, and the gel poured into a column (5 x 9 cm). The column was washed with 150 mL of buffer containing 25 mM β-glycerophosphate, 10 mM MOPS, 2 mM EDTA, 2 mM sodium vanadate, 1 mM DTT, pH 7.2 and eluted with 800 mL of a 0.0-0.4 M NaCl linear gradient.

Fractions with kinase activity (ie, that phosphorylated myelin basic protein) were pooled, diluted 1:1 with buffer C (12.5 mM β-glycerophosphate, 12.5 mM MOPS, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 0.05 mM NaF, 2 mM DTT) and loaded onto a hydroxylapatite column (2.5 x 5 cm). The column was washed with buffer C and eluted with a 300 mL 0.0-0.35 M potassium phosphate linear gradient. Active kinase fractions capable of phosphorylating myelin basic protein were pooled, diluted 1:3 with buffer C and loaded onto a phosphocellulose column (2.5 x 5 cm). The column was washed with buffer C and the total volume collected, as the kinase did not bind to this matrix.

The phosphocellulose fraction was loaded onto a phenyl-Sepharose column (2.5 x 6 cm), washed with buffer C and eluted with a 300 mL 0.0-0.3% Brij-35 linear gradient in buffer C. Active fractions capable of phosphorylating myelin basic protein were pooled and loaded onto a heparin-Sepharose column (2.5 x 5 cm). The kinase activity did not bind to this matrix, and was collected in the eluate. This eluate was loaded onto a polylysine-agarose column (2.5 x 6 cm), the column washed, and proteins eluted with 300 mL 0.0-0.7 M NaCl linear gradient in buffer C. Active fractions were pooled, diluted 1:3 with buffer E and loaded onto a Mono-Q column (1 mL bed volume). The kinase activity was eluted with a 30 mL 0.0-0.8 M NaCl linear gradient in buffer C. The active fractions were pooled, concentrated and stored at -70 ^{o}C.

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CHAPTER 3 - SOLUBLE SKELETAL MUSCLE EXTRACTS AS CRUDE PROTEIN KINASE PREPARATIONS TO PHOSPHORYLATE DYSTROPHIN'S CARBOXYL TERMINUS

INTRODUCTION

Dystrophin is present in terminally differentiated muscle cells (Lev *et al* 1987), and is phosphorylated *in vivo* in primary rat skeletal muscle cell cultures (Milner *et al*, 1993). Therefore mature muscle should be a good source of tissue to characterize and identify the kinases involved in the phosphorylation of dystrophin. The carboxyl terminus of dystrophin is believed to be one of the most functionally significant domains of the dystrophin molecule. This region of the protein also contains consensus sites for several protein kinases (figure 1-2). The ability of crude protein kinase preparations, made from soluble skeletal muscle extracts and cell culture extracts, to phosphorylate the carboxyl terminus of dystrophin was investigated.

Reversible phosphorylation, as a postranslational protein modification, plays an essential role in the regulation of intracellular events. It controls significant events such as membrane transport, contractility, cell metabolism, transcription, gene expression, cell division, myogenesis, fertilization, memory, intra- and extra-cellular signalling and protein-protein interactions, to name a few. Phosphorylation can occur on a variety of residues including lysine, arginine and histidine, but most commonly on serine, threonine, and tyrosine. Some phosphorylation events are structural in nature, in that the phosphate group is not removed from the substrate protein. Most other events, however, involve a dynamic fluctuation between the phosphorylated and dephosphorylated state. The enzymes responsible for the dephosphorylated state are protein phosphatases, which are only now becoming recognized for their critical role in regulatory events. In some cases, the true regulation of an intracellular phosphorylation event and the subsequent behavior modification could be based upon the activity of the phosphatase, and not the protein kinase.

Dystrophin is a protein of low abundance, representing approximately 0.0002% of the total skeletal, cardiac or smooth muscle protein, and approximately 0.00002% of the total protein in brain tissue (Hoffman *et al.*, 1987). It maintains a tight membrane association, mediated by a membrane-bound glycoprotein complex, during purification. It is very difficult to purify in large or usable quantities; therefore we have expressed a glutathione-S-transferase fusion protein, named GST-CT-1, containing the carboxyl 201 amino acids of dystrophin, residues 3485-3685, (Milner *et al.*, 1992). Fusion protein construction obviates the problem of isolating native dystrophin from membranes and of purifying this low-abundance protein. The value of fusion proteins has been previously demonstrated in functional studies, showing actin binding by dystrophin's amino terminal domain (Hemmings *et al.*, 1992; Way *et al.*, 1992), and Ca^{++} binding to the cysteine-rich region (Milner *et al.*, 1992).

The source of muscle for the soluble extracts included rat and rabbit skeletal muscle. The muscle cell cultures employed in these experiments were also of rat origin. Though the muscle tissue and muscle cell sources are from two different species, they are both mammalian, and were found to behave similarly with regards to kinase activity against dystrophin. The dystrophin substrate was a protein produced in *E. coli* from a human skeletal cDNA.

Using the GST-CT-1 dystrophin fusion protein as a substrate, I have discovered at least two different protein kinase activities in soluble skeletal muscle and cellular extracts. Their characterization demonstrates differences in the way that they may be regulated, as well as differences in their preferred sites of phosphate incorporation into the carboxyl terminus of dystrophin. Attempts were made to partially purify and identify the protein kinases.

RESULTS

I. Use of a dystrophin fusion protein to study dystrophin phosphorylation

I investigated whether soluble extracts prepared from mature skeletal muscle, as well as soluble extracts prepared from cell lines and primary cell culture, could be used as protein kinase sources to study dystrophin phosphorylation. The recombinant GST-CT-1 dystrophin fusion protein was employed to study phosphorylation of the carboxyl terminus.

Figure 3-1a shows the mobility of the purified dystrophin GST-CT-1 fusion protein (lane 1) and GST alone (lane 2). Note that the purified GST-CT-1 fusion protein is present as a doublet. The upper band has an apparent molecular weight of 44 kDa and the lower, 41 kDa. The upper band was identified with the anti-dystrophin peptide antibody (figure 3-1b, lane 1) which recognizes a sequence (see figure 1-2) which is present in the last 18 amino acids of dystrophin's carboxyl terminus. The lower protein band was not recognized by this antibody and must therefore be missing the epitope. Both the upper and the lower protein band interacted with antibodies directed against a protein A CT-1 fusion protein (PA-CT-1) (figure 3-1b, lane 2). Also recognized by this antibody were proteins ranging in between 31 and 42 kDa. Based upon immunological identification with these antibodies, they were most likely proteolytic fragments of the dystrophin fusion protein, with carboxyl truncations. This antibody recognized only the dystrophin sequences in GST-CT-1. Anti-GST antibodies also recognized the upper and lower protein bands of the GST-CT-1 fusion protein (including proteolytic fragments of the dystrophin fusion protein), as well as the purified GST protein (figure 3-1b, lanes 3 and 4). This analysis demonstrated that the lower protein band was a truncated fragment of the upper protein band, and that it was missing the antigenic site for the anti-dystrophin peptide antibody which is contained in the last 18 amino acids of the dystrophin protein. The cause of this anomaly is not clear, but it was most likely due to a hypersensitive proteolytic site in the carboxyl terminus of dystrophin. The upper band and the lower band are referred to throughout this thesis, as full length GST-CT-1 and truncated GST-CT-1, respectively.

II. Different protein kinase activities are present in the soluble skeletal muscle extracts

Using the GST-CT-1 fusion protein as a substrate, it was established that the skeletal muscle extract contained at least two different protein kinase activities that phosphorylate the carboxyl terminus of dystrophin under two mutually exclusive conditions (presence or absence of Ca^{++}) (Figure 3-2a). Muscle extracts were prepared in Buffer A in the absence or presence of Ca^{++},

Figure 3-1. Identification of the dystrophin fusion protein, GST-CT-1

GST-CT-1 and GST were expressed in *E. coli*, purified and subjected to SDS-PAGE on 12.5% gels and immunological analysis as described under "Methods" sections III, IV and II. (a) Coomassie blue staining, lane 1, purified GST-CT-1; lane 2, purified GST. (b) Immunostaining, lane 1, GST-CT-1 immunostained with anti-dystrophin peptide antibodies; lane 2, GST-CT-1 immunostained with anti-PA-CT-1 antibodies; lane 3, GST-CT-1 immunostained with anti-GST antibodies; lane 4, GST immunostained with anti-GST antibodies. Five µg of protein were loaded per lane. GST (glutathione-S-transferase), GST-CT-1 (glutathione-S-transferase-carboxyl terminal-1). The positions of the molecular weight marker proteins are indicated. The arrows indicate full length GST-CT-1, truncated GST-CT-1 or GST. c) Schematic representation of the full length and carboxyl-truncated GST-CT-1. The arrow indicates the antibody recognition site where the amino acid sequence used to synthesize the peptide is located.

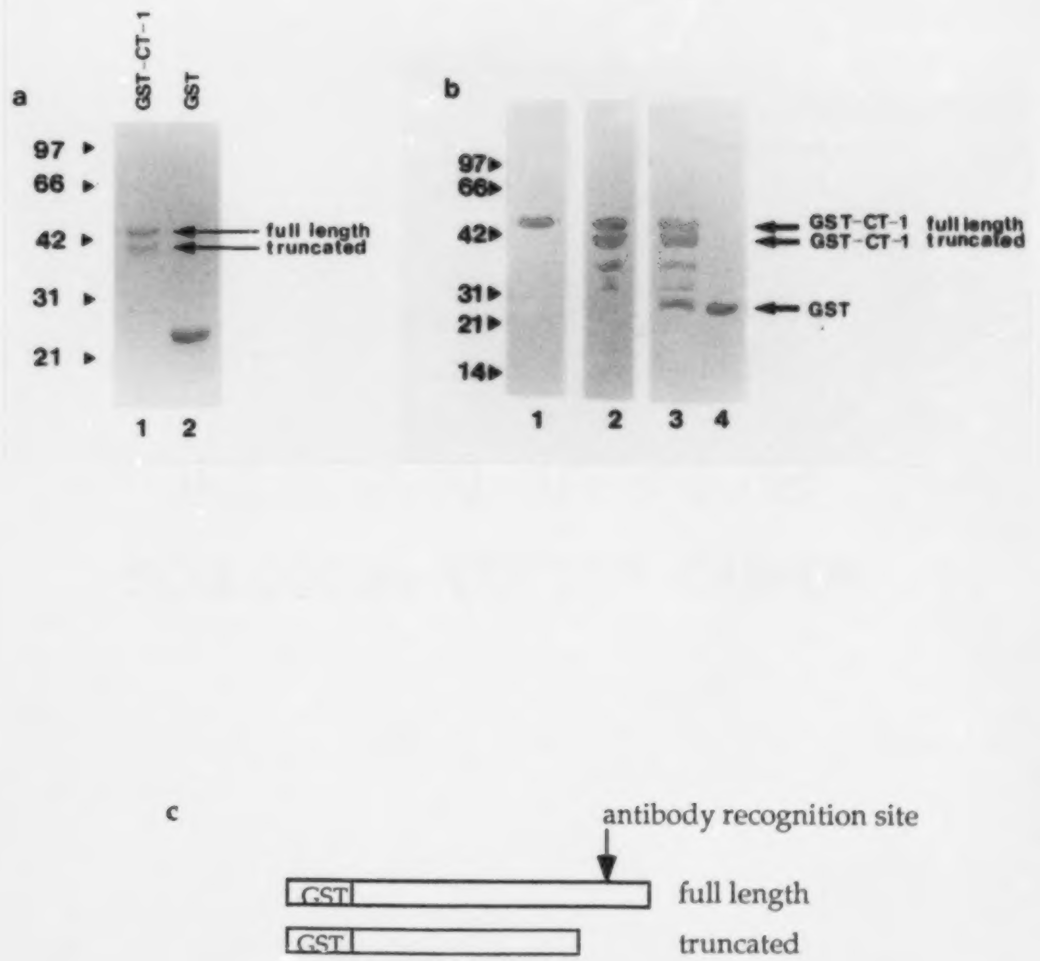
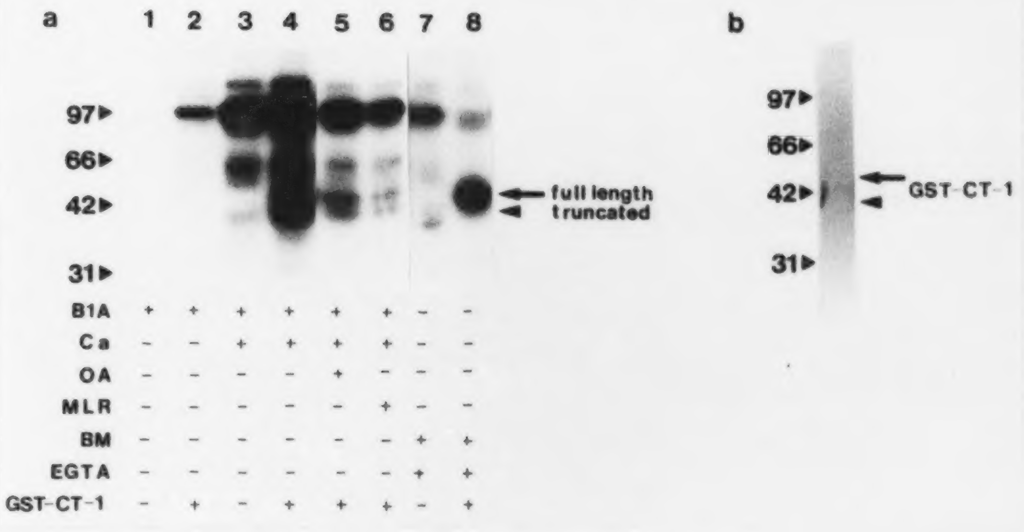

a
GST-CT-1
GST
97
66
42
31
21
full length
truncated
1
2
b
97
66
42
31
21
14
GST-CT-1 full length
GST-CT-1 truncated
GST
1
2
3
4
c
antibody recognition site
GST
full length
GST
truncated

Figure 3-2. Examination of the protein kinase activities in soluble skeletal muscle extracts which phosphorylate the carboxyl terminus of dystrophin

Soluble skeletal muscle extracts were prepared from rabbit skeletal muscle and liver tissues, dialyzed against Buffer A (B1A) (which consisted of 50 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM PMSF, 1 mM benzamidine and a cocktail of protease inhibitors), or Buffer B (BM) (which consisted of 12.5 mM Mops pH 7.2, 7.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM DTT, 0.5 mM EGTA, 0.05 mM NaF, 1 mM PMSF, 1 mM benzamidine and a cocktail of protease inhibitors), and used for phosphorylation of GST-CT-1. Phosphorylation of GST-CT-1 was investigated with the independent additions of 2 mM Ca^{++} (Ca), 20 nM okadaic acid (OA) or 20 nM microcystin LR (MLR). Pretreatment with any of these compounds was carried out for 10 min at room temperature before the phosphorylation reaction of 30 min at 30 ^{o}C. The reactions were terminated and the proteins subjected to SDS-PAGE on 10% gels and autoradiographed as described under "Methods" VI, VIII and II. a) Phosphorylations were carried out in the presence (+) or absence (-) of GST-CT-1 in Buffer A (B1A), lanes 1 and 2; Buffer A plus 2 mM Ca^{++} (Ca), lanes 3-6; or Buffer B (BM) lanes 7 and 8. Reactions (lanes 5 and 6) were preincubated with either 20 nM okadaic acid (OA) or microcystin LR (MLR) for 10 min at room temperature. b) Liver extracts were dialyzed in Buffer A and used to phosphorylate GST-CT-1 in the presence of 2 mM Ca^{++}. Thirty μg of skeletal muscle and liver extract, and 5 μg of GST-CT-1 were loaded per lane. The arrow indicates full length GST-CT-1 and the arrowhead truncated GST-CT-1. Positions of the molecular weight marker proteins are indicated.


a
1 2 3 4 5 6 7 8
97▸
66▸
42▸
31▸
full length
truncated
B1A + + + + + + − −
Ca − − + + + + − −
OA − − − − + − − −
MLR − − − − − + − −
BM − − − − − − + +
EGTA − − − − − − + +
GST-CT-1 − + − + + + − +
b
97▸
66▸
42▸
31▸
GST-CT-1

or in Buffer B in the absence of Ca^{++}. Figure 3-2a, lanes 1-6 are GST-CT-1 phosphorylated by skeletal muscle extracts in Buffer A in the presence or absence of 2 mM Ca^{++}. Lane 4 shows the significant increase in phosphotransferase activity that was observed in the presence of 2 mM Ca^{++}. This demonstrates that the muscle extracts contained a Ca^{++}-dependent protein kinase activity which phosphorylated both the full length and truncated GST-CT-1. Figure 3-2a, lane 5 shows that the phosphorylation of GST-CT-1 by this Ca^{++}-stimulated protein kinase was reduced in the presence of 20 nM okadaic acid, an inhibitor of protein phosphatases 1 and 2A (Bialojan and Takai 1988). The addition of 20 nM mycrocystin-LR (lane 6), which also inhibits protein phosphatases 1 and 2A (MacKintosh *et al*, 1990), caused a decrease in the amount of phosphate incorporated into GST-CT-1. The effect of microcystin LR was greater than that observed for okadaic acid. Figure 3-2a, lanes 7-8 show GST-CT-1 phosphorylated by skeletal muscle extracts in the presence of Buffer B containing 0.5 mM EGTA. Phosphate is incorporated primarily in the full length GST-CT-1. Under these conditions neither protein kinase activity phosphorylated GST (data not shown). In order to test the specificity of muscle extracts for the phosphorylation of dystrophin, extracts were also prepared from nonmuscle tissue. Figure 3-2b demonstrates that liver extracts did not phosphorylate the carboxyl terminus of dystrophin.

Results of these experiments indicate that the soluble skeletal muscle extracts may have contained at least two separate protein kinase activities: 1) an okadaic acid sensitive, Ca^{++}-stimulated activity, hereafter named protein kinase XA, which phosphorylates both full length and truncated GST-CT-1 and is active in Buffer A plus Ca^{++}; and 2) an activity, hereafter named protein kinase XB, which phosphorylates only the full length GST-CT-1, and is active in Buffer B (containing 0.5 mM EGTA). Each of these activities may represent one or more protein kinases.

III. Characterizations of Protein kinase XA and Protein kinase XB

Though the identification of protein kinases XA and XB remains inconclusive, their behavior in skeletal muscle extracts in response to the addition of specific compounds was investigated for further characterization. Characterization was performed using rabbit and rat skeletal extracts, which were found to behave similarly with regards to the phosphorylation of dystrophin (data not shown). Protein kinase XA activity was reduced by okadaic acid and heparin, while stimulated by Ca^{++}, Zn^{++}, protein kinase inhibitor (PKI) and polylysine (figure 3-3a). Protein kinase XB activity was reduced by Mn^{++}, Ca^{++} and heparin. None of the compounds tested stimulated the activity of protein kinase XB (figure 3-3b). These results demonstrate further differences between the two protein kinase activities present in skeletal extracts which phosphorylate dystrophin's carboxyl terminus.

Phospho-amino acid analysis of the full length dystrophin fusion protein demonstrated that protein kinase XA incorporated phosphate into a serine residue(s) (figure 3-3c).

1V. Is GST-CT-1 phosphorylated by purified casein kinase II?

The carboxyl terminus of dystrophin contains several different consensus sites for casein kinase II (CKII), (figure 1-2). In order to determine if these sites are relevant *in vitro*, GST-CT-1 was tested in a phosphorylation reaction with CKII purified from bovine brain. Several documented substrates exist for CKII, which besides casein include both skeletal and cardiac calsequestrin (Cala and Jones 1991), and phosphatase inhibitor-2. Figure 3-4 displays autoradiographs of the substrates subjected to a phosphorylation reaction with the purified CKII. Negative controls include recombinant (lane 2) and native pancreatic calreticulin (lane 4). The proteins successfully phosphorylated are the known substrates cardiac and skeletal (lane 5 and 7) calsequestrin, phosphatase inhibitor-2 (lane 6) and casein (not shown). This bovine CKII did not find GST-CT-1 a suitable substrate.

V. Partial purification of protein kinase XA

V-1. Ammonium sulfate fractionations of the skeletal muscle extracts

In order to further characterize and partially purify the protein kinase XA activity(s), soluble skeletal muscle extracts were fractionated with ammonium sulfate into 25%, 45% and 65% saturated supernatants. Collected fractions were tested for their ability to phosphorylate GST-CT-1 and their sensitivity to okadaic acid. Figure 3-5 shows that protein kinase activity was

Figure 3-3. Characterization of the protein kinase activities in soluble skeletal muscle extracts which phosphorylate the carboxyl terminus of dystrophin

Rabbit and rat skeletal muscle extracts were prepared, dialyzed against Buffer A (rabbit) or Buffer B (rat), and used for phosphorylation of GST-CT-1. Samples were preincubated with the additions indicated (lanes 3-10) for 10 min at room temperature before the addition of substrate and $\gamma[^{32}P]$ATP. The phosphorylation reactions were incubated for 30 min at 30 ^{o}C, terminated and the proteins were subjected to SDS-PAGE and autoradiographed as described under "Methods" sections VI, VIII and II. a) Phosphorylation in Buffer A plus 2 mM Ca^{++}, b) phosphorylation in Buffer B. Lane 3, 2 μM okadaic acid (OA); lane 4, 2 mM Mn^{++} (Mn); lane 5, 2 μM okadaic acid and 2 mM Mn^{++}; lane 6, 50 μM Ca^{++}; lane 7, 50 μM Zn^{++}; lane 8, 130 mg/mL protein kinase inhibitor (PKI); lane 9, 0.1 μM polylysine (PLys); lane 10, 2 μg/mL heparin (Hep). Sixty μg of extracts and 5 μg of GST-CT-1 were employed in each reaction. The arrow indicates full length GST-CT-1 and the arrowhead truncated GST-CT-1. The position of the molecular weight protein markers are indicated. c) Full length GST-CT-1 was phosphorylated by extracts made in Buffer A plus 2 mM Ca^{++}, and subjected to acid hydrolysis followed by high voltage electrophoresis as described in "Methods". Lane 1, phospho-amino acid standards stained with ninhydrin (light areas); lane 2, autoradiograph of the phosphorylated amino acid(s) of GST-CT-1. The arrowhead indicates phosphoserine, and the arrow the direction of electrophoresis.

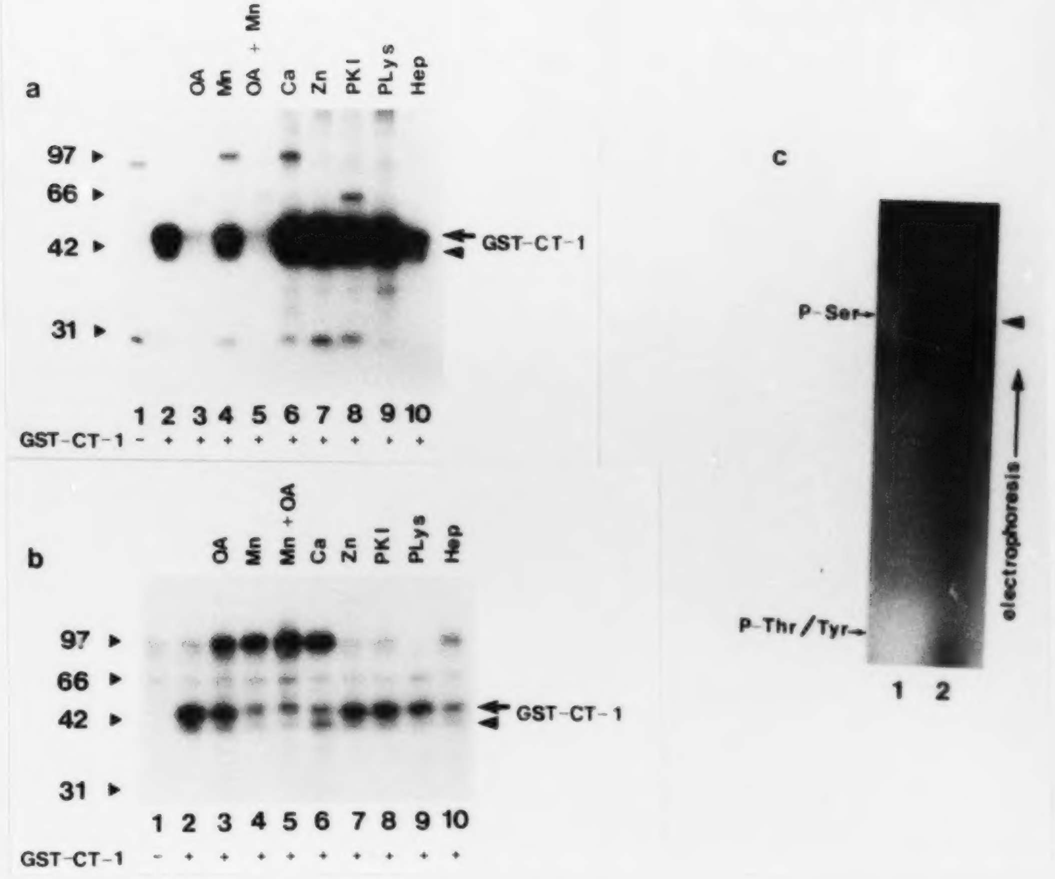

a
OA
Mn
OA + Mn
Ca
Zn
PKI
PLys
Hep
97
66
42
31
GST-CT-1
1 2 3 4 5 6 7 8 9 10
GST-CT-1 - + + + + + + + + +
b
OA
Mn
Mn + OA
Ca
Zn
PKI
PLys
Hep
97
66
42
31
GST-CT-1
1 2 3 4 5 6 7 8 9 10
GST-CT-1 - + + + + + + + + +
c
P-Ser
P-Thr / Tyr
electrophoresis
1 2

Figure 3-4. Substrate specificity of the purified CKII

The casein kinase was purified as described, and used to phosphorylate various substrates as described under "Methods" sections XIV, VIII and II. Purified CKII was used to phosphorylate GST-CT-1 (lane 1), recombinant calreticulin (RCRT) (lane 2), canine pancreatic calreticulin (DPCRT) (lane 4), canine cardiac calsequestrin (CCCS) (lane 5), phosphatase inhibitor-2 (I-2) (lane 6), and rabbit skeletal calsequestrin (RSCS) (lane 7). Autophosphorylation of CKII was investigated in lane 3. Five µg of protein were added per lane. The positions of the molecular weight marker proteins are indicated.

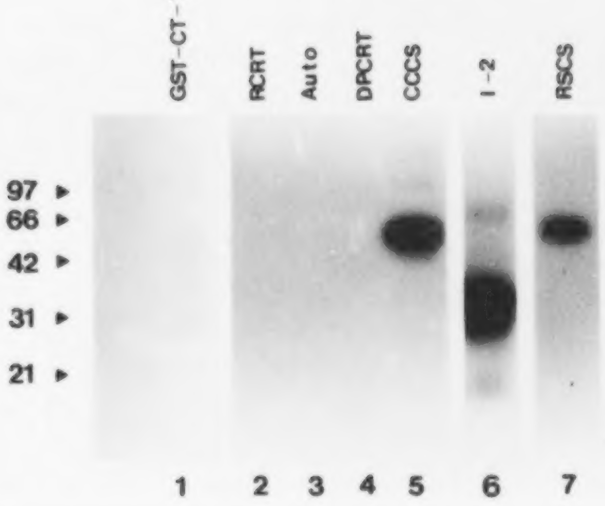
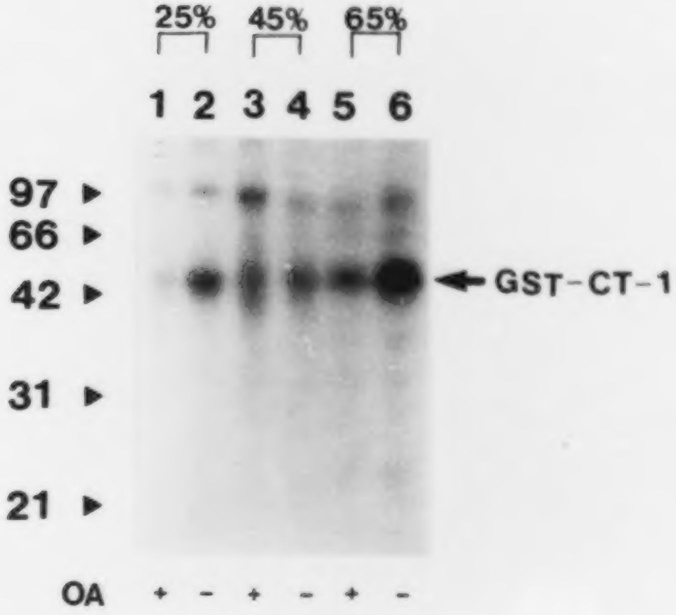

GST-CT-1
RCRT
Auto
DPCRT
CCCS
I-2
RSCS
97 ▸
66 ▸
42 ▸
31 ▸
21 ▸
1
2
3
4
5
6
7

Figure 3-5. Okadaic acid sensitivity of the ammonium sulfate fractions of soluble skeletal muscle extract

Skeletal muscle extracts were prepared in Buffer A plus 2 mM Ca^{++}, fractionated with ammonium sulfate into 25%, 45% and 65% ammonium sulfate fractions, dialyzed in Buffer A plus 2 mM Ca^{++}, and used to phosphorylate GST-CT-1. The phosphoproteins were subjected to SDS-PAGE on 12.5% gels and autoradiographed, all as described in the "Methods" sections VI, VIII and II. Phosphorylation of GST-CT-1 was carried out in the presence (+) or absence (-) of 2 μM okadaic acid after a 10 min preincubation period at room temperature. Five μg of GST-CT-1 and 60 μg of extract were loaded in each lane. GST-CT-1 exists as a single protein band in these experiments. The positions of the molecular weight protein markers are indicated.


25%
45%
65%
1
2
3
4
5
6
97
66
42
31
21
GST-CT-1
OA
+
-
+
-
+
-

present in all of the ammonium sulfate fractions, but only the activity present in the 45% ammonium sulfate fraction was insensitive to okadaic acid.

V-2. Behavior of the Soluble Skeletal Muscle Extract on a p13 Chromatographic Affinity Column

It has been documented in our laboratory that the carboxyl terminus of dystrophin is phosphorylated *in vitro* by protein kinase $p34^{cdc2}$ (Milner *et al*, 1993). Members of the cdc-like family of protein kinases can be purified by affinity chromatography employing p13, a protein product of the yeast suc1 gene believed to play a role in cell division (Hayles *et al*, 1986; Hindley *et al*, 1987; Brizuela *et al*, 1987). To determine if the protein kinase(s) which phosphorylate dystrophin's carboxyl terminus bind to p13, the extracts were incubated with a p13-coupled CNBr-Sepharose column. The beads were washed and used in a phosphorylation reaction as a source of protein kinase activity. Figure 3-6 shows that though protein kinases were present in the original extract, and the supernatant, the p13-Sepharose did not retain any active protein kinase(s) which phosphorylated GST-CT-1.

VI. Use of the cellular extracts as crude protein kinase preparations in the phosphorylation of the carboxyl terminus of dystrophin

Differentiation of muscle cells is physically characterized by fusion of the mononucleated myoblasts into multinucleated myotubes. Two rat muscle cell lines, L6 and Sol8, and a rat primary muscle cell culture were employed to study the GST-CT-1-phosphotransferase activity during myogenesis. Under the conditions of these studies, fusion of myoblasts began at day 4 (after seeding) for L6 cells, day 3 for Sol8 cells and day 3 for the primary cell culture. Dystrophin is expressed at the time of fusion (Lev *et al*, 1987; Klamut *et al*, 1989). Extracts were prepared from the cell lines and primary cultures at each day of a time course. These extracts were then tested for their ability to phosphorylate GST-CT-1 using Buffer B or Buffer A containing 2 mM Ca^{++} (Figure 3-7).

All cell lines tested displayed an increase in the GST-CT-1 phosphotransferase activity at or near myoblast fusion into myotubes, under conditions of either Buffer A containing Ca^{++} (figure 3-7d), or Buffer B (figure 3-7a,b,c). It is important to note, however, that the protein kinase activity in

Figure 3-6. Partial purification of the soluble skeletal muscle extracts using a p13 affinity column

The p13 protein was produced, purified and coupled to CNBr-activated Sepharose. Soluble skeletal muscle extracts, dialyzed against Buffer A containing 2 mM Ca^{++}, were incubated with the p13 beads. Material bound to p13 was screened for the ability to phosphorylate GST-CT-1. Comparative phosphorylation reactions using untreated muscle extract and the supernatant from the p13 incubation were prepared, and the proteins were separated on SDS-PAGE and autoradiographed as described under "Methods" sections X, VI, VIII and II. Ex (skeletal muscle extract), Supt (unbound extract proteins), p13-Seph (p13-Sepharose-bound proteins). The arrow indicates full length GST-CT-1 and the arrowhead truncated GST-CT-1. The positions of the molecular weight protein markers are indicated.

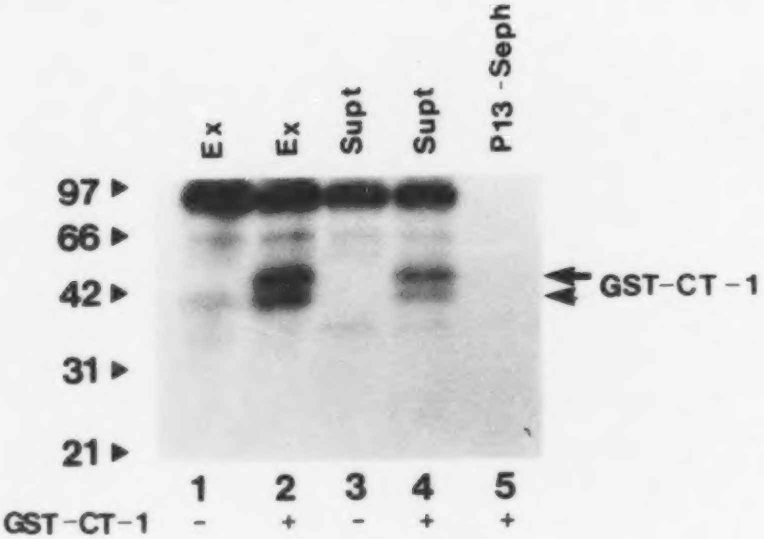
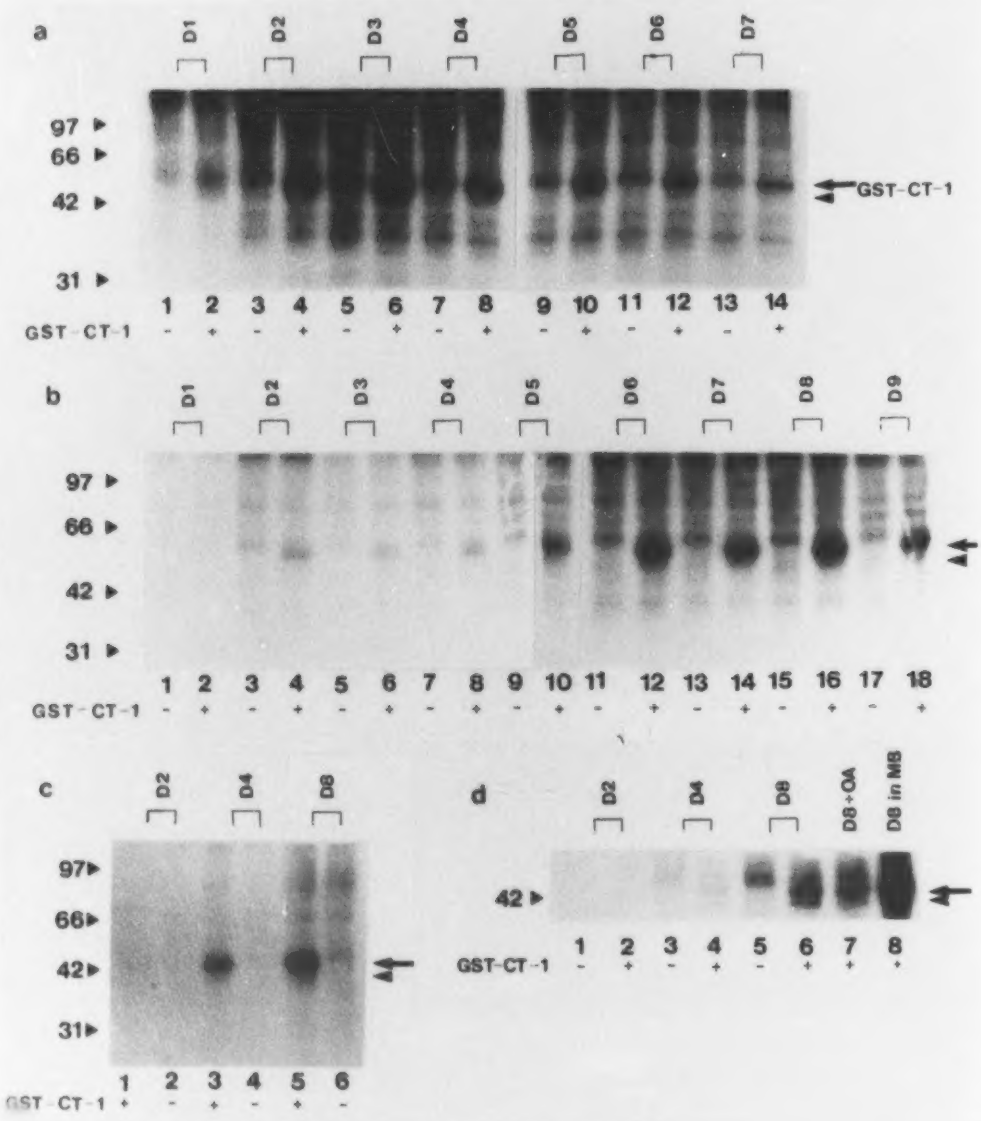

Ex
Ex
Supt
Supt
P13 -Seph
97
66
42
31
21
GST-CT-1
1
2
3
4
5
GST-CT-1
-
+
-
+
+

Figure 3-7. Phosphorylation of GST-CT-1 by cell culture extracts

Rat muscle cell lines (Sol8, L6) and rat primary skeletal muscle cell cultures were prepared, maintained and allowed to fuse. Extracts were prepared from cells on each day of growth, and were used to phosphorylate GST-CT-1 as crude protein kinase preparations in the presence of Buffer B (a, b, c) or Buffer A plus 2 mM Ca^{++} (d), as described under "Methods" sections XIII, VIII and II. (a) L6 extracts in the absence (-) and presence (+) of GST-CT-1, days 1-7 (D1-D7). (b) Sol8 extracts in the absence (-) and presence (+) of GST-CT-1, days 1-9 (D1-D9). (c) Primary cell culture extracts in the absence (-) and presence of GST-CT-1, day 2, 4 and 8 (D2-D8). (d) Buffer A/Ca^{++}. Lanes 1-6 as above for figure 3-6c. Lane 7 in the presence of 2 μM okadaic acid (OA). Lane 8, Buffer B (MB). Twenty μg of protein from each extract was added to each lane The positions of the molecular weight marker proteins are shown. GST-CT-1 existed as a doublet in these experiments. The arrow is full length GST-CT-1, and the arrowhead is truncated GST-CT-1.



a
D1
D2
D3
D4
D5
D6
D7
97
66
42
31
GST-CT-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
GST-CT-1 - + - + - + - + - + - + - +
b
D1
D2
D3
D4
D5
D6
D7
D8
D9
97
66
42
31
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
GST-CT-1 - + - + - + - + - + - + - + - + - +
c
D2
D4
D8
97
66
42
31
1 2 3 4 5 6
GST-CT-1 + - + - + -
d
D2
D4
D8
D8+OA
D8 in MB
42
1 2 3 4 5 6 7 8
GST-CT-1 - + - + - + + +

the L6 cell line declined with time in culture (figure 3-7a, lanes 7-14). The protein kinase activities in Sol8 and primary cell culture, however, did not decrease after fusion (figure 3-7b, lanes 13-18; figure 3-7c, lanes 5 and 6; figure 3-7d, lanes 5 and 6).

The protein kinase that was active in Buffer A containing 2 mM Ca^{++} was tested only in primary skeletal muscle cell culture extracts (figure 3-7d). The protein kinase activity incorporated phosphate into the full length and truncated GST-CT-1. This activity was also sensitive to okadaic acid (figure 3-7d, lane 7). The Ca^{++} activation, pattern of phosphate incorporation and okadaic acid sensitivity were very similar to that described for protein kinase XA present in mature skeletal muscle (figure 3-2, lanes 3-6). The protein kinase that was active in Buffer B phosphorylated only the full length GST-CT-1 and not the truncated GST-CT-1. The pattern of phosphate incorporation was similar to that observed for protein kinase XB (figure 3-2a, lane 8) found in mature skeletal muscle extracts.

DISCUSSION

In this series of experiments, I have presented evidence suggesting that the carboxyl terminus of dystrophin, as represented by CT-1, is a substrate of protein kinases in soluble skeletal muscle extracts. There are at least two different unidentified protein kinase activities (protein kinase XA and protein kinase XB) (see table 3-1) in these muscle extracts. It was also demonstrated, using soluble extracts from muscle cell cultures, that protein kinases which phosphorylate the carboxyl terminus of dystrophin are active at the time of myotube formation.

I. The location of phosphate incorporation

The GST-CT-1 fusion protein employed in these studies existed primarily as two proteins (figure 3-1). The presence of the truncated fragment provided a fortuitous method of phosphopeptide analysis. Based on immunological identification, it is concluded that the smaller fragment contains at least 18 amino acids less than its intact parent molecule (figure 3-1c). This fragmentation allowed localization of phosphate incorporation for each protein kinase to either the first 184 dystrophin amino acids or the last 18

Table 3-1. Protein kinase activities present in soluble skeletal muscle extracts

Buffer A +2mM Ca (protein kinase XA)	Buffer B (protein kinase XB)
Phosphorylation of full length and truncated GST-CT-1	Phosphorylation primarily of full length GST-CT-1
Activity was reduced in the presence of okadaic acid	Activity unaffected by the presence of okadaic acid
Activated by Ca^{++}	Activity reduced in the presence of Ca^{++}
Phospho-serine	Untested
Peak of activity at or near cell fusion	Peak of activity at or near cell fusion
Present in tissue and cells	Present in tissue and cells

amino acids of full length GST-CT-1. This was determined by observing if there were any apparent differences in labelling between the two protein bands.

Comparison of figure 3-2 (lanes 4 and 8) demonstrates that the two unidentified activities, kinases XA and XB, phosphorylated the dystrophin fusion protein on different sites. Protein kinase XA incorporated labelled phosphate into the first 184 dystrophin amino acids of the GST-CT-1 fusion protein, as evidenced by nearly equal labelling of both full length and truncated GST-CT-1. Protein kinase XB incorporated labelled phosphate primarily in the full length GST-CT-1, which can be interpreted to mean that the site(s) is located within approximately the last 18 amino acids of the molecule.

II. The effect of okadaic acid and mycrocystin LR addition to the muscle extracts

Phosphorylation events are often dynamic in nature, with dephosphorylation of the substrate proteins carried out by phosphatases. The effects of specific inhibitors of these phosphatases on the phosphorylation of proteins provide information about how the substrate phosphorylation state is being controlled, and possibly how the protein kinase itself is regulated. There are several different types and subtypes of protein phosphatases present in skeletal muscle (for review see Cohen 1989), including protein phosphatases type 1 (PP-1), which is ATP- and magnesium-dependent; PP-2A, which is stimulated by cations; PP-2B, which is Ca^{++}- and calmodulin-dependent; and PP-2C, which is magnesium-dependent.

Okadaic acid and mycrocystin LR are protein phosphatase inhibitors specific for PP-1 and PP-2A. Okadaic acid, a polyether produced by marine plankton (dinoflagellates), inhibits PP-1 in the low nM range and PP-2A 100 fold more strongly in the sub-nM range (Cohen *et al./*, 1990). Microcystin LR, a cyclic heptapeptide produced by cyanobacteria, inhibits both phosphatases nearly equally in the nM range (MacKintosh *et al*, 1990). Their addition is usually employed to prevent substrate dephosphorylation when the phosphorylation reaction is carried out in an extract. An expected result of their presence would be the increase in phosphate incorporation into a substrate, because of the protection from phosphatases, and the therefore unopposed protein kinase activity (Sassa *et al* 1989). This was found not to be the case in these studies.

The addition of okadaic acid (figure 3-2, lane 5; figure 3-3a, lane 3) or microcystin LR (figure 3-2, lane 6) to skeletal muscle extracts reduced the amount of labelled phosphate incorporation into GST-CT-1 by protein kinase XA. There are two possible interpretations of the observed inhibition (or reduction of phosphate incorporation into the substrate) of protein kinase XA. 1) The protein kinase itself is inhibited by okadaic acid (or microcystin LR). This cannot be tested until there is purified protein kinase available. This possibility is, however, unlikely, in that so far okadaic acid has had no effect on any one of over eight protein kinases tested (Takai *et al*, 1987; Suganuma *et al*, 1988; Haystead *et al* 1989). 2) The protein kinase itself is regulated by phosphorylation/dephosphorylation events, and is active when dephosphorylated (Figure 3-8). Figure 3-2 (lanes 5 and 6) reveals that microcystin LR reduced phosphate incorporation into substrate to an even greater extent than okadaic acid. Based upon the reported levels of phosphatase sensitivity, this implies that a type 1 protein phosphatase may be responsible for regulation of protein kinase XA.

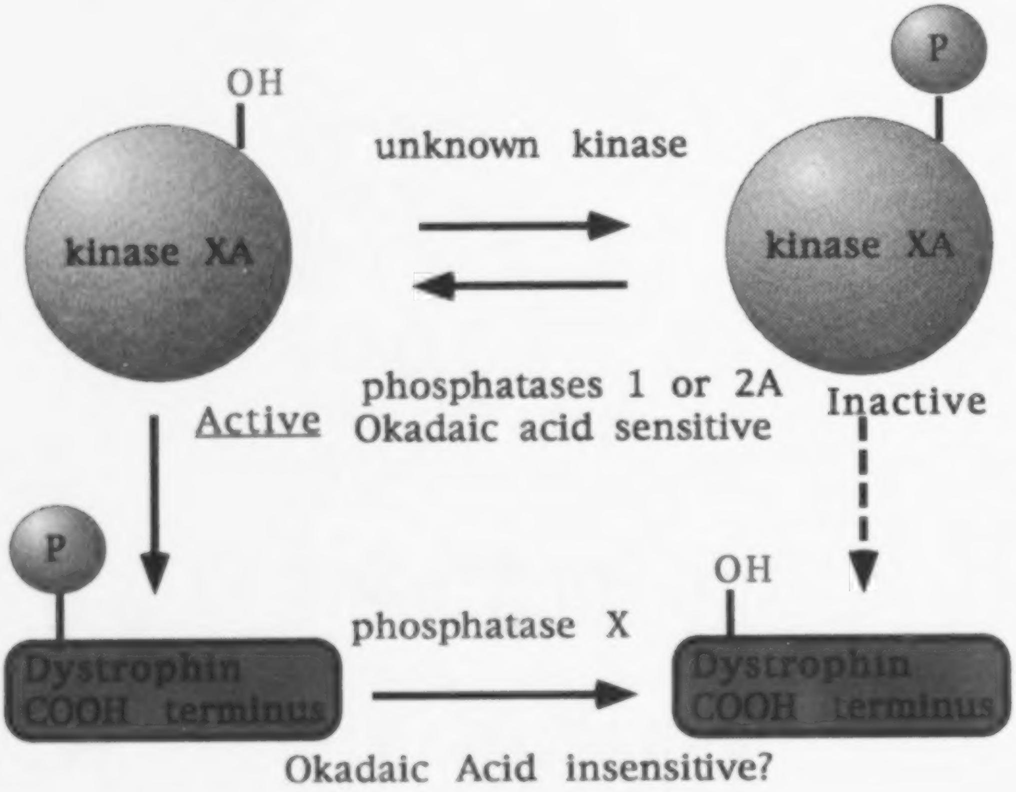
Protein kinase XB appeared to be insensitive to okadaic acid (figure 3-3b, lane 3), which implies that it is a different protein kinase which is not regulated in the same manner as protein kinase XA. The small decrease in phosphate incorporation observed (figure 3-3b, lane 3) could be due to competiton of reaction components with the unknown 97 kDa muscle substrate which was also phosphorylated under these conditions.

III. Further characterization of kinases XA and XB

Additional characterization of these two protein kinase activities (XA and XB) by various compounds (figure 3-3) demonstrated more differences. Metal ions such as Mn^{++}, Ca^{++} and Zn^{++} have been known to activate protein phosphatases (Cohen, 1989; Wagner and Mutus, 1991). Protein kinase XA activity was stimulated by Ca^{++} and Zn^{++} (figure 3-3a, lanes 6 and 7). These metal ions could be 1) activating the protein kinase and/or 2) activating a phosphatase, possibly PP-2B (Wagner and Mutus, 1991), which positively

Figure 3-8. A possible mechanism for the effect of okadaic acid upon the phosphorylation of GST-CT-1 by the muscle extract.

Protein kinase XA may be active when dephosphorylated. In the extract there are assumed to be regulatory kinases and phosphatases. When okadaic acid (or microcystin LR) is added to the extract, the phosphatases are inhibited and the balance of the reaction proceds in the direction of inactivating protein kinase XA due to its unopposed phosphorylation by an 'unknown kinase'. The balance of regulatory control of protein kinase XA could possibly be shifted by the addition of purified protein phosphatase 1 and/or 2A.


OH
kinase XA
unknown kinase
P
kinase XA
phosphatases 1 or 2A
Okadaic acid sensitive
Active
Inactive
P
Dystrophin
COOH terminus
OH
phosphatase X
Dystrophin
COOH terminus
Okadaic Acid insensitive?

regulates protein kinase XA. Given the insensitivity of PP-2B to okadaic acid (Cohen *et al*, 1989), it is more likely that these ions are stimulating the protein kinase directly. Mn^{++} had a very small, negative effect on the incorporation of phosphate into GST-CT-1 by protein kinase XA (figure 3-3a, lane 4). Zn^{++} had little effect (figure 3-3b, lane 7), but Ca^{++} and Mn^{++} (figure 3-3b, lanes 6 and 4) caused a reduction in the phosphate incorporation into GST-CT-1 by protein kinase XB. These metals may 1) inhibit the protein kinase(s) directly or indirectly and/or 2) activate a phosphatase that acts to dephosphorylate the substrate GST-CT-1.

These results also demonstrate that there were at least two activities present in the protein kinase XB preparation, as Ca^{++} selectively reduced the activity which transfers phosphate to the last 18 amino acids of the dystrophin sequence (full length GST-CT-1). There remained another activity, which phosphorylated the fusion protein in its first 184 dystrophin amino acids, as seen by incorporation into both protein bands of the fusion protein.

An interesting observation was the phosphorylation of a 97 kDa muscle protein present in the skeletal muscle extracts (figure 3-2). The protein appeared to be phosphorylated by a Ca^{++} activated kinase, in a way that was similar to that seen with the GST-CT-1 substrate. However, the 97 kDa protein was also seen to be phosphorylated in lane 2, where the only difference between it and lane 1 where the protein is not phosphorylated, is the presence of GST-CT-1. These results may suggest that either GST-CT-1 is itself a kinase, or that the fusion proteins is somehow stabilizing the responsible kinase. The identity of the 97 kDa muscle protein is unknown, but based on its size and source, could be phosphorylase.

IV. Is protein kinase A involved in phosphorylating dystrophin's carboxyl terminus?

The response of the kinases in the extracts to other chemical factors such as protein kinase A (PKA) inhibitor (PKI) was examined (figure 3-3). Protein kinase XA experienced an increase and protein kinase XB possibly a small decrease in activity. The increase in phosphate incorporation into GST-CT-1 by the addition of PKI may suggest that PKA is involved in a negative regulatory cascade for protein kinase XA, and that this cascade may be much larger that the one indicated in figure 3-8.

V. Could a casein kinase be involved in phosphorylating dystrophin's carboxyl terminus?

Heparin is a potent competitive inhibitor of CKII (Feige *et al* 1980), whereas polyamines (Mäenpää, 1977) and basic polypeptides such as polylysine (Meggio *et al* 1983, Meggio and Pinna, 1989, Palen and Traugh 1991) are known to activate the enzyme.

There are many clues that suggest that either protein kinase XA or XB may be a casein kinase-like enzyme. Protein kinase XA activity was enhanced by polylysine (figure 3-3a, lane 9) and reduced by heparin (figure 3-3a, lane 10). It is interesting to note that, although heparin inhibition was evident for protein kinase XB (figure 3-3b, lane 10), there was no polylysine stimulation of that activity (figure 3-3b, lane 9). Protein kinase XA activity demonstrated both casein kinase-like phenomena quite well. This could mean that protein kinase XB is not a true casein kinase, but that it may share some regulatory properties. It is also possible that the amount of heparin present was affecting protein kinase XA or XB in a non-regulatory fashion, and may therefore not be of the casein kinase family.

The carboxyl terminus of dystrophin contains several different consensus sites for casein kinase II (CKII), (figure 1-2). In order to determine if these sites are relevant *in vitro*, GST-CT-1 was tested in a phosphorylation reaction with CKII purified from bovine brain (figure 3-4). Unfortunately, this bovine CKII did not find GST-CT-1 a suitable substrate. This does not rule out the possibility that CKII isolated from muscle could phosphorylate GST-CT-1, nor that a casein kinase-like activity could be active in the extracts.

VI. Is a Ca^{++}/calmodulin-activated protein kinase involved in the phosphorylation of dystrophin's carboxyl terminus?

Protein kinase XA activity was stimulated by Ca^{++}, which implies that the protein kinase is potentially a Ca^{++}-calmodulin-dependent protein kinase (CaM kinase). Preliminary data from another project demonstrated that the dystrophin fusion protein was phosphorylated to a stoichiometry of 1.5 on two sites by CaM kinase II. The full length GST-CT-1 was phosphorylated on serine(s) and threonine(s), and the truncated GST-CT-1 on serine(s) (Shemanko, Walsh and Michalak). However, CaM kinases are often positively regulated by autophosphorylation, and protein kinase XA activity appears to be positively regulated by dephosphorylation. It is possible that the extract conditions are providing an incomplete view of the protein kinase regulation. It is also possible that the protein kinase XA activity is a novel isoform of CaM kinase II.

VII. Partial purification of protein kinase XA from soluble skeletal muscle extracts

Partial purification of protein kinase XA was attempted by using ammonium sulfate fractionation (figure 3-5), and p13 affinity chromatography (figure 3-6). Figure 3-5 demonstrates that different fractions of the muscle extract displayed different sensitivities to okadaic acid. This could be interpreted as 1) there was more than one activity present, or 2) that one activity was spread out in one or more fractions and that the regulatory subunit(s) of these activities were dissociated from the catalytic subunit. Protein kinases belonging to the cdc2 family bind p13. The p13 affinity column demonstrated that protein kinase(s) XA did not bind p13 or that it was inactived under these conditions.

VIII. The use of cell line and primary skeletal muscle tissue culture extracts to study the phosphorylation of the carboxyl terminus of dystrophin

Skeletal muscle cells do not divide. They are multinucleated myotubes formed by fusion of mononucleated myoblasts. Myoblasts are capable of proliferating and differentiating. This system provided an opportune method to study skeletal muscle differentiation, because myoblast proliferation and ultimate fusion to myotubes can be controlled by factors present in the medium. The absence of growth factors is permissive for fusion. There are considerable differences in the protein profile between myoblasts and myotubes which is indicative of muscle-specific gene expression (Devlin and Emerson, 1978; Lev *et al* 1987, and Miranda *et al* 1988; Endo and Nadal-Ginard 1987; Bader *et al* 1982; Chamberlain *et al* 1985; Jaynes *et al* 1986; Klarsfield *et al* 1987).

Figure 3-7 shows that the carboxyl portion of dystrophin was phosphorylated by protein kinase activity in various cellular extracts taken at the time of, and after, fusion. It appears that the protein kinase activities in the cell lines were those of protein kinase XA (3-7d) and XB (figure 3-7a, 3-7b, 3-7c) found in adult muscle extracts, based on the phosphorylation conditions, locations of phosphate incorporation and okadaic acid sensitivity. Phosphopeptide and phosphoamino acid analysis could demonstrate this definitively. The increase in cell line protein kinase XA and -XB activity over time suggests that perhaps they are not expressed, or are inactive, or active at low levels, until the time of fusion. Dystrophin itself is not visible at the plasma membrane until after fusion in culture (Lev *et al*, 1987; Klamut *et al*, 1989). The presence of these activities after fusion, and in adult skeletal muscle tissue, reveals that these kinases are important and active in differentiated cells. It is possible that the phosphorylation of dystrophin's carboxyl terminus is important during myogenesis and it may control the localization of dystrophin to the plasma membrane, or the protein-protein interactions essential for its correct localization and function.

In conclusion, this study has demonstrated that skeletal muscle extracts and cell extracts contain kinases capable of phosphorylating the carboxyl terminus of dystrophin, as represented by CT-1. There are at least two different protein kinases that phosphorylate dystrophin, on at least two different sites under mutually exclusive conditions. Protein kinase XA is Ca^{++} activated, possibly positively regulated by PP-1 and/or PP-2A, and transfers phosphate to the first 184 amino acids of the dystrophin sequence present in the fusion protein GST-CT-1. Protein kinase XB is active in the absence of Ca^{++} and transfers phosphate to approximately within the last 18 carboxyl amino acids of dystrophin. There appears to be additional, minor protein kinase activity present in each categorized activity. These two activities, kinases XA and XB, could represent opposing behavioral control of dystrophin under differing levels of cellular Ca^{++}.

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CHAPTER 4

PHOSPHORYLATION OF THE CARBOXYL-TERMINAL OF DYSTROPHIN BY MITOGEN-ACTIVATED PROTEIN (MAP) KINASE*

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CHAPTER 4 - PHOSPHORYLATION OF THE CARBOXYL TERMINUS OF DYSTROPHIN BY MITOGEN-ACTIVATED PROTEIN KINASES

INTRODUCTION

Inspection of the sequence of the entire dystrophin molecule has indicated that it contains a number of consensus sites for phosphorylation by different kinases. Interestingly, within the carboxyl-terminal 201 amino acids of skeletal muscle dystrophin there are at least four consensus sites for phosphorylation by MAP kinase (mitogen-activated protein kinase). MAP kinase belongs to the family of the proline directed kinases. In addition to the consensus sites for MAP kinase, there are also two consensus sites for phosphorylation by the protein kinase $p34^{cdc2}$ in the carboxyl-terminal region of dystrophin. Together, these sequences suggest that member(s) of the family of proline directed protein kinases may be involved in phosphorylation of dystrophin. In keeping with this, we have shown that both native dystrophin (in isolated sarcolemmal vesicles) and recombinant fragments of the carboxyl-terminal of dystrophin are substrates for phosphorylation by the human $p34^{cdc2}$ protein kinase, *in vitro* (Milner *et al.*, 1993).

In this study, we show that the carboxyl-terminal of dystrophin is phosphorylated (~1 mole P/mole of protein) *in vitro* by MAP kinase, at a threonine residue(s). In addition, we show that the carboxyl-terminal domain of dystrophin is phosphorylated by protein kinase(s) in soluble extracts of skeletal muscle. Further, when the muscle extracts are fractionated by Mono-Q FPLC chromatography, fractions with protein kinase activity against dystrophin correspond to the fractions containing MAP kinase (identified by activity against myelin-basic protein and immunological reactivity).

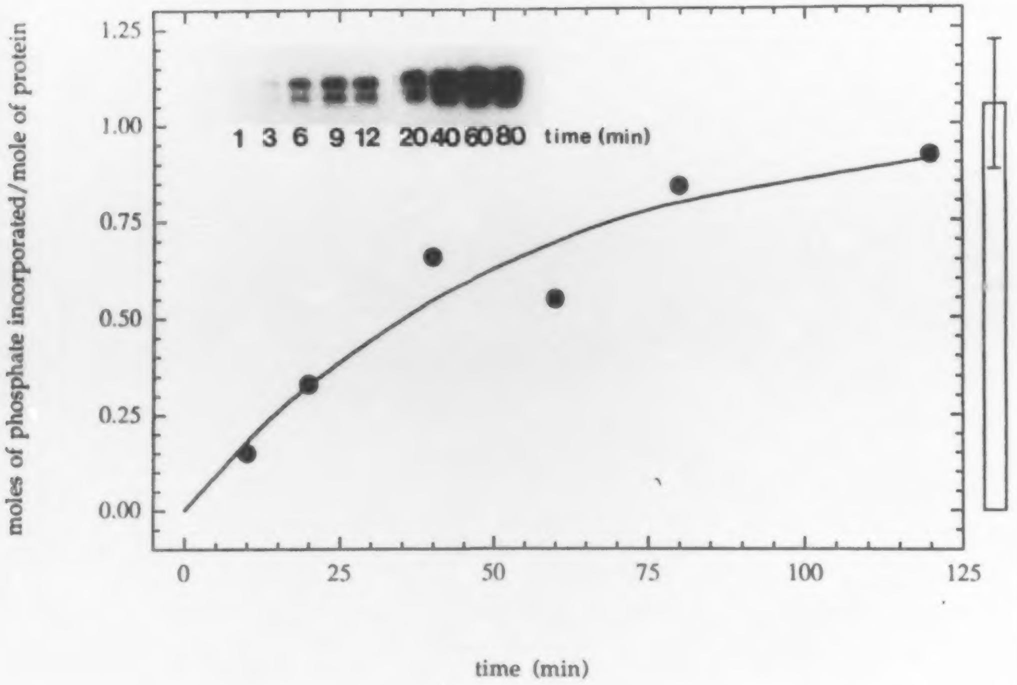
RESULTS

I. Phosphorylation of the carboxyl-terminal region of dystrophin by MAP kinase

Incubation of the purified GST-CT-1 with MAP kinase (purified from sea star oocytes) led to incorporation of ^{32}P into both protein bands (figure. 4-1). In contrast, recombinant GST was not phosphorylated by MAP kinase (data not shown). The level of ^{32}P incorporation was apparently unaffected by

Figure 4-1. Phosphorylation of GST-CT-1 by sea star MAP kinase $p44^{mpk}$

Phosphorylation of the GST-CT-1 by purified sea star $p44^{mpk}$ was carried out for the time indicated as described under "Methods" part XV, VIII and II. The inset is an autoradiograph of GST-CT-1 phosphorylated over the time indicated. The graph represents quantification of phosphate incorporated into GST-CT-1 from a separate experiment. Quantification was performed by precipitation of the phosphorylated proteins on Whatman 3MM paper pretreated with trichloroacetic acid and sodium tungstate. The bar in B indicates stoichiometry of the phosphorylation - 1.00 ± 0.16 mole P/mole of protein. Two and one half μg of GST-CT-1 were loaded per lane.


moles of phosphate incorporated/mole of protein
1.25
1.00
0.75
0.50
0.25
0.00
0
25
50
75
100
125
time (min)
1 3 6 9 12 20 40 60 80 time (min)

proteolysis of the fragment CT-1. A time course for the incorporation of ^{32}P into GST-CT-1 was determined (figure. 4-1), from which the maximal incorporation was estimated to be 1.00 ± 0.16 mole of P/mole of protein (plateau calculated using Sigma Plot).

II. Phosphorylation of the carboxyl terminal of dystrophin by soluble muscle extracts

Dystrophin is phosphorylated *in vivo* in skeletal muscle primary cell cultures (Milner *et al.*, 1993). In this study we isolated a soluble extract (100,000 x g supernatant) from mature rabbit skeletal muscle in order to detect and identify any kinases that might phosphorylate dystrophin. Figure 4-2 illustrates the phosphorylation of recombinant GST-CT-1 that was observed as a result of incubation with the soluble muscle extract. The figure shows that the soluble fraction of skeletal muscle contained kinase(s) that phosphorylate a variety of proteins in the extract itself, and that one or more of these kinases also phosphorylate the carboxyl-terminal region of dystrophin.

III. Phosphoamino acid analysis and phosphopeptide mapping

Phosphoamino acid analysis was carried out in order to further characterize the phosphorylation of GST-CT-1 by both purified MAP kinase and by the muscle extract. In both cases, the protein was phosphorylated selectively on threonine residue(s) (Figure 4-3, B and D). Further, the phosphopeptide maps obtained for GST-CT-1 after phosphorylation with the pure MAP kinase and with the muscle extract revealed very similar patterns (Figure 4-3, A and C). The only observed difference between the two peptide maps was the presence of a few additional radiolabelled peptides after phosphorylation with the muscle extract (Figure 4-3 C). The phosphopeptide maps suggest that MAP kinase was present in the muscle extracts and that it was active against GST-CT-1. The maps also indicate that the muscle extract contained additional kinase(s) that phosphorylated the carboxyl-terminal region of dystrophin.

IV. Fractionation of muscle extracts by FPLC chromatography

To confirm the presence of MAP kinase in the muscle extracts and to demonstrate its activity against dystrophin, muscle extracts were fractionated

Figure 4-2. Phosphorylation of GST-CT-1 by soluble skeletal muscle extracts

Rabbit skeletal muscle extracts were prepared in extraction buffer (10 mM dibasic sodium phosphate pH 7.5, 60 mM b-glycerophosphate, 15 mM EGTA, 15 mM MgCl2, 1 mM DTT, 10 mM PMSF, 1mM benzamidine, and a 1 in 2000 dilution of a protease inhibitor cocktail) in the presence of 1 µM okadaic acid without further dialysis, and used to phosphorylate GST-CT-1 as described in "Methods" section VI, VIII and II. Lane 1, 5 µg of GST-CT phosphorylated by crude extracts; lane 2, 6 µg of rabbit soluble skeletal muscle extract phosphorylated without additional substrate. Thirty µg of muscle extract and 5 µg of GST-CT-1 were added per lane. The arrow indicates full length GST-CT-1 and the arrowhead truncated GST-CT-1. Positions of the molecular weight marker proteins are indicated.

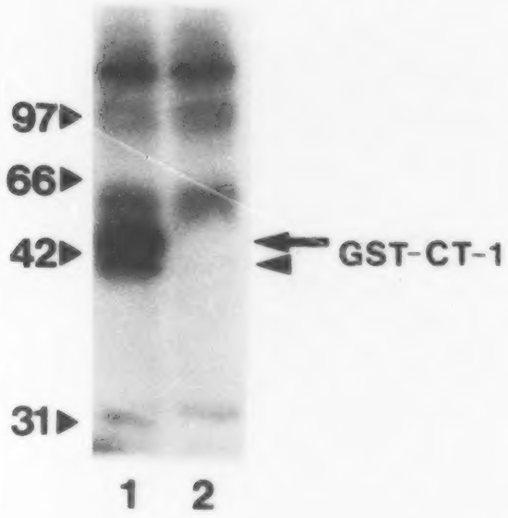
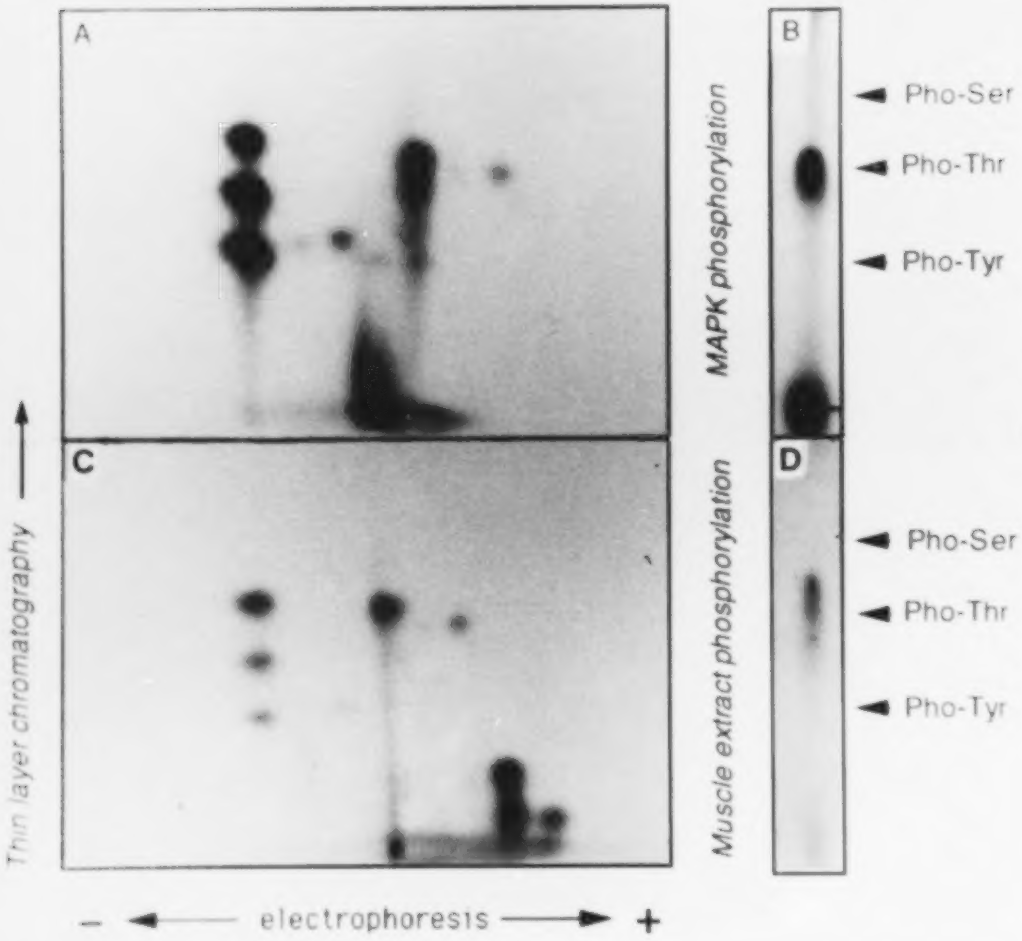

97▶
66▶
42▶
31▶
GST-CT-1
1
2

Figure 4-3. Phosphopeptide and phosphoamino acid analysis of GST-CT-1

GST-CT-1 was phosphorylated by purified MAP kinase or rabbit skeletal muscle extracts. The labeled proteins were either digested with trypsin or acid hydrolyzed, and subjected to two dimensional mapping for phosphopeptide and phosphoamino acid analysis respectively. These phosphopeptide and phosphoamino acid analysis were carried out in Dr. S. Pelech's laboratory as described under "Methods" section IX, and VIII. A, B - MAP kinase phosphorylation; C, D - muscle extract phosphorylation.


A
B
C
D
Pho-Ser
Pho-Thr
Pho-Tyr
Pho-Ser
Pho-Thr
Pho-Tyr
MAPK phosphorylation
Muscle extract phosphorylation
Thin layer chromatography
− electrophoresis +

by Mono Q chromatography. Fractions were tested for kinase activity against GST-CT-1 and against myelin basic protein, an identified substrate for MAP kinase. Fractions were also screened for the presence of MAP kinase(s) using specific antibodies against two isoforms of the protein, $p44^{erk1}$ and $p42^{mapk}$. Figure 4-4 shows that the skeletal muscle extracts contain a peak of kinase activity against both myelin basic protein and GST-CT-1 (Figure. 4-4A), and that the shoulder of activity against GST-CT-1 is coincident with the peak of activity against myelin basic protein and immunologically detected MAP kinase $p44^{erk1}$ (Figure. 4-4B).

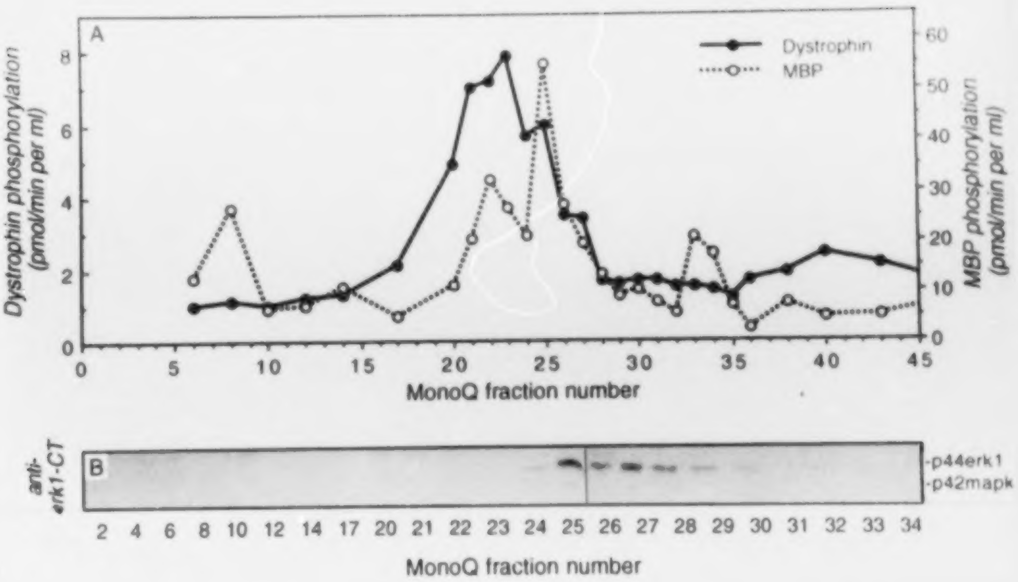
DISCUSSION

In this part of my research we have shown that the carboxyl terminal region of dystrophin (CT-1) is an *in vitro* substrate for stoichiometric phosphorylation by MAP kinase. Further, we have established that a soluble fraction isolated from mature skeletal muscle contains kinases that can phosphorylate CT-1; and on the basis of phosphopeptide maps and phosphoamino acid analysis we conclude that one of these is MAP kinase ($p44^{erk1}$ and $p42^{mapk}$).

The consensus site for MAP kinase is PX(Ser or Thr)P (Clark-Lewis et al., 1991), where X is a basic or neutral amino acid for $p42^{mapk}$ and $p44^{erk1}$. p44mpk will tolerate an acidic residue in the X position. The consensus site for $p34^{cdc2}$ is (Z or X)(Ser or Thr)PXZ where X is a polar residue and Z is a basic one (Moreno and Nurse, 1990). There are four potential phosphorylation sites for MAP kinase in the carboxyl-terminal 201 amino acids of skeletal muscle dystrophin; Ser^{3490}, Thr^{3548}, Ser^{3549}, and Ser^{3552}. In this study, both purified MAP kinase and the MAP kinase(s) in skeletal muscle extracts phosphorylated CT-1 at threonine residue(s) only. The most likely site of incorporation would be at residue Thr^{3548}, a slightly unconventional site of incorporation (Pro-Thr*-Ser-Pro). In a previous study, we have shown that the carboxyl terminal region of dystrophin is a substrate for *in vitro* phosphorylation by human cyclin-dependent protein kinase $p34^{cdc2}$, at both serine and threonine residues (Milner *et al.*, 1993). The threonine residue is in a different location from the threonine residue employed as a phosphoacceptor by MAP kinase. It is possible that this reflects

Figure 4-4. Fractionation of the skeletal muscle extract by Mono-Q FPLC chromatography

A soluble rabbit skeletal muscle extract prepared in extraction buffer in the presence of okadaic acid was fractionated in Dr. S. Pelech's laboratory on a Mono-Q column, and screened as described under "Methods" section VI, VII and II. Fractions were screened by phosphorylation of either GST-CT-1 or myelin basic protein (A). The fractions were also size separated by SDS-PAGE, transferred to nitrocellulose membrane and screened for reactivity with anti-erk1-CT antibodies.


A
Dystrophin
MBP
Dystrophin phosphorylation (pmol/min per ml)
MBP phosphorylation (pmol/min per ml)
0
2
4
6
8
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
45
MonoQ fraction number
anti-erk1-CT
B
-p44erk1
-p42mapk
2 4 6 8 10 12 14 17 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
MonoQ fraction number

a difference in function of these sites, and that MAP kinase phosphorylation of dystrophin may regulate the function of dystrophin independently of $p34^{cdc2}$ or its related kinases. As yet, the functional significance of these observations cannot be determined because the function of dystrophin is unknown. However, as dystrophin can be phosphorylated *in vivo* (Milner *et al.*, 1993), the results generated here suggest that proline directed kinases may have some role in this.

$p34^{cdc2}$ kinase and MAP kinase belong to a family of proline directed protein kinases. Both kinases are M-phase activated ($p34^{cdc2}$ kinase, Moreno and Nurse, 1990; MAP kinase, Tamemoto *et al.*, 1992; Sanghera *et al.*, 1990), as well as present in differentiated tissue ($p34^{cdc2}$ kinase, Meyerson *et al.*, 1992; MAP kinase, Boulton *et al.*, 1991). The expression of the MAP kinase gene has been shown to increase during muscle differentiation (Boulton *et al.*, 1991). In this study we immunologically identified an active MAP kinases, $p44^{erk1}$, in the soluble fraction of rabbit skeletal muscle. Therefore, MAP kinase is also active in fully differentiated cells, and likely has functions other than those involved in cell cycle progression. MAPK has been shown to be involved in the control of many protein-protein interactions (Gille *et al.*, 1992; Childs *et al.*, 1992). In some instances, control of cytoskeletal behavior appears to be under dual regulation by MAP kinase and $p34^{cdc2}$ (Shiina *et al.*, 1992; Childs *et al.*, 1992; Yamashiro *et al.*, 1990; Yamashiro *et al.*, 1991; Mak *et al.*, 1991). Dystrophin is a cytoskeletal protein which has been shown to be dually phosphorylated by both MAP kinase and $p34^{cdc2}$.

It is possible that phosphorylation of the carboxyl-terminal of dystrophin by proline directed kinase(s) may regulate its interaction with the DAPs. Direct testing of this hypothesis awaits cloning and expression of the glycoproteins. Binding studies, and/or reconstitution experiments could then be used to study the interaction between the glycoproteins and recombinant domains of dystrophin.

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Footnotes

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[b] Medical Research Council Scientists

[c] Scholar of the Alberta Heritage Foundation for Medical Research. Correspondence should be addressed to this author.

CHAPTER 5 - GENERAL DISCUSSION AND CONCLUSIONS

It was established in this study that the carboxyl terminus of dystrophin is a substrate for multisite phosphorylation by at least three protein kinase activities in mature and developing skeletal muscle (figure 5-1).

1) Kinase XA is activated by calcium, positively regulated by protein phosphatases types 1 and 2A, (perhaps most specifically PP1), and phosphorylates the dystrophin fusion protein on a serine residue(s) within the first 184 amino acids.

2) Kinase XB is active in the absence of calcium and it is regulated differently than kinase XA. This activity phosphorylates the fusion protein within approximately the last 18 amino acid residues. In addition, there may be one or more kinase activities that phosphorylate dystrophin present in the two kinase preparations.

3) Purified MAP kinases $p44^{erk}$ and $p42^{mpk}$ (active when protected from deactivation by PP1 or PP2A), phosphorylate the dystrophin fusion protein on a threonine residue(s) in the first 184 amino acids.

Though kinase activities XA and XB were partially characterized in these studies, their identities remain unclear. It is possible, however, that a calcium/calmodulin (CaM) activated protein kinase contributes to the kinase XA activity. Preliminary results demonstrate that the dystrophin fusion protein is also phosphorylated by purified CaM kinase II on two separate sites (Chapter 3, section VI). It is necessary to identify the corresponding activity in the extracts, and characterize its significance *in vitro* and *in vivo*.

I. Functional significance of dystrophin phosphorylation

Dystrophin has been proposed to function in one or more of three possible ways: 1) to be involved in membrane stability, 2) to be responsible for linking the contractile force to the external evironment, 3) to be involved in organizing membrane topology or maintaining positions of membrane components. The observed phosphorylation of dystrophin may play a role in these, or other functions.

Table 5-1. Protein kinase activities present in soluble skeletal muscle extracts

Buffer A	Buffer A +2mM Ca (protein kinase XA)	Buffer B (protein kinase XB)
Phosphorylation of both bands*	Phosphorylation of full length and truncated GST-CT-1	Phosphorylation of full length GST-CT-1
*Activity only in extracts prepared with OA (PP1 and 2A inhibitor)	Activity is reduced in the presence of okadaic acid	Activity unaffected by the presence of okadaic acid
	Activated by Ca^{++}	Activity reduced in the presence of calcium
Phosphothreonine	Phosphoserine	Untested
	Peak of activity at or near cell fusion	Peak of activity at or near cell fusion
MAP kinase		
Present in tissue, cells untested	Present in tissue and cells	Present in tissue and cells

For example, phosphorylation of dystrophin could control its interactions with other proteins. Dystrophin interacts with DAPs via its cysteine-rich region and the carboxyl terminus (Suzuki *et al*, 1992). It is possible that phosphorylation of dystrophin's carboxyl terminus could play a role in mediating these interactions. It has been demonstrated that dystrophin is phosphorylated by protein kinase $p34^{cdc2}$ (Milner *et al*, 1993) and MAP kinase (this study). These kinases control cytoskeletal protein-protein interactions both independently and dually. Research into the functional significance of dystrophin's phosphorylation by $p34^{cdc2}$, MAP kinase and the two unknown skeletal muscle kinases is critical to understanding not only their role, but that of dystrophin itself. It is therefore quite possible that dystrophin is phosphorylated on multiple sites to control different aspects of its function.

II. The pattern of dystrophin's consensus sites are conserved

Dystrophin shares some of its carboxyl terminal consensus sites with several of its related proteins. Dystrophin-related proteins include the 70-75 kDa alternative DMD protein product, utrophin (DRP), and the 87 kDa *Torpedo* protein. Many of the sites are conserved in a form of dystrophin found in *Torpedo* electric organ post-synaptic membrane. The MAP kinase consensus sites and their locations are conserved to some extent in each of these proteins (Table 5-2). The MAP kinase site, PTSP, which contains two phosphoacceptors Thr-3548 or Ser-3549, of which the threonine is significant, is completely conserved only in the 71 kDa protein. A skeleton MAP kinase site PTP is present at that location in the 87 kDa protein. Conservation or alteration of these sites could imply functional diversity or similarity between these related proteins.

Of the remaining consensus sites within the carboxyl terminus of dystrophin, very few others are conserved in the related proteins, with the exception of those in the 71 kDa protein. This protein contains all of dystrophin's carboxyl terminus kinase consensus sites except for the very last $p34^{cdc2}$ protein kinase, CaM kinase and CKI sites.

Table 5-2. Conservation of Dystrophin's Kinase Consensus Sites in Related Proteins

Phosphorylation sites in proteins related to dystrophin are indicated by (+) if the site is completely conserved, and (-) if it is not conserved. Partial conservation is designated by the altered amino acid sequence in brackets.

Dystrophin Consensus Sites		*Torpedo* Dystrophin	71 kDa protein	87 kDa protein	DRP (utrophin)
GSK III	Ser-3486	+	+	+ (SSQQRAP)	+
	Ser-3537	+	+	-	-
MAP kinase	Ser-3490	+	+	-	+
	Thr-3548	-	+	+ (PTP)	-
	Ser-3549	+ (PISP)	+	-	-
	Ser-3552	+	+	-	-
CK II	Ser-3541	+	+	-	+
	Ser-3552	+	+	-	-
	Ser-3639	-	+	-	-
	Ser-3653	+ (SSELED)	+	-	-
	Thr-3654	-	+	-	-
p34^{cdc2}	Ser-3549	+	+	-	-
	Thr-3675	+ (DTPGKK)	-	-	-
CaM kinase	Ser-3623	+	+	-	-
	Thr-3684	-	-	-	-
CK I	Thr-3684		-	-	-

III. *Torpedo* electric organ 87 kDa protein

The fact that the related 87 kDa *Torpedo* protein has been discovered to be phosphorylated on serine, threonine and tyrosine residues, suggests that the phosphorylation of dystrophin is significant. This 87 kDa protein is highly homologous to dystrophin's carboxyl terminus, and is believed to be present in the neuromuscular junction of muscle cells. Dystrophin itself also exists in the *Torpedo* electric organ post-synaptic membrane. *Torpedo* dystrophin has been found to associate as a complex with at least two other proteins, a 58 kDa and an 87 kDa protein, with the 58 kDa protein mediating the interactions. They were found to associate in the *Torpedo* post-synaptic membrane by co-purification (Butler *et al* 1992) and coimmunoprecipitation (Wagner *et al* 1993). These proteins are believed to have mammalian skeletal muscle counterparts (Butler *et al* 1992). The cloned protein shows that it contains sequences similar to the cysteine-rich domain and carboxyl terminus of human skeletal dystrophin (Wagner *et al* 1993). Most interestingly, the 87 kDa protein is phosphorylated on serine, threonine and tyrosine residues. It has been suggested that it functions in the formation and maintenance of synapses and is regulated by protein phosphorylation. The 87 kDa protein may form a link between integral membrane proteins such as the nicotinic acetylcholine receptor and the membrane cytoskeleton, similar to the putative role of *Torpedo* dystrophin. This suggests that phosphorylation of the carboxyl terminus of dystrophin may play some role in its interactions with the membrane.

In the future, there are several areas for investigation. 1) As already discussed, it is imperative to isolate and identify the kinase activities present in skeletal muscle that are responsible for phosphorylating dystrophin's carboxyl terminus and the remaining portions of the dystrophin molecule *in vivo*. 2) It is also critical to establish functional significance for each event, if possible. However, this work is restricted by the lack of functional assays available for dystrophin. It was discovered that phosphorylation was not involved in protein interactions of GST-CT-1 with any proteins present in soluble and TX-100-solubilized skeletal muscle extracts (data not shown). However, phosphorylation may control its interactions with membrane proteins. The carboxyl 201 amino acids of dystrophin are able, when expressed in cell culture, to localize to the plasma membrane successfully (Shemanko, Busaan and Michalak). It is possible that phosphorylation could control this behavior. Deletion constructs or site directed mutagenesis of known phosphorylation sites could be used in transformation experiments to test this possibility. 3) Identification of the phosphatases involved in regulating dystrophin's phosphorylation would also be an important achievement in understanding the complexities of the regulation of dystrophin function is regulated. 4) Physical-chemical studies such as circular dichroism could be employed to determine if there are any physical effects of phosphorylation upon dystrophin. This study awaits either very pure dystrophin in large quantities, or a recombinant protein expressed with a very small fusion protein.

IV. Conclusions

1) This study has conclusively identified the carboxyl terminus of dystrophin as a substrate for phosphorylation by kinases present in skeletal muscle extracts.

2) At least three major protein kinase activities phosphorylate multiple sites of the carboxyl terminus of dystrophin, including two partially characterized activities, kinases XA and XB, and a MAP kinase, $p44^{erk1}$.

3) This multisite phosphorylation indicates that dystrophin may require complex and precise regulation.

4) Specific phosphorylation of the carboxyl-terminal region of dystrophin by MAP kinases suggests that this protein may respond to stimuli in the extracellular environment, further supporting its putative role as a communication mediator.

Further identification of the other kinases, characterization of their *in vivo* and functional significance will contribute to the establishment of dystrophin's essential cellular role.

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